REGULATION OF HISTONE GENE TRANSCRIPTION DURING THE CELL CYCLE IN HeLa S_3 CELLS

By

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KEY TO ABBREVIATIONS

BME	Eagle's basal media
Cr_0t	concentration of ribonucleotides x time
EDTA	ethylene diamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane-sulfonic acid
POPOP	1,4-di(2-(5 phenyloxazoyl))benzene
PPO	2,5 diphenyloxazole
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate (150 mM NaCl, 15 mM Na citrate, pH 7.0)
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl ethylene diamine
T_m	melting temperature

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

REGULATION OF HISTONE GENE TRANSCRIPTION DURING THE CELL CYCLE IN HeLa S_3 CELLS

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In order to directly examine the availability of histone gene for transcription, chromatin from both G_1 and S phase cells was transcribed *in vitro* with *E. coli* RNA polymerase and the ability of the transcripts to form S_1 nuclease resistant TCA precipitable hybrids with histone cDNA was determined. In initial studies in this laboratory, it was shown that histone sequences can be transcribed *in vitro* from chromatin isolated from S phase cells but that chromatin isolated from G_1 phase cells does not serve as a template for the transcription of histone sequences. In experiments in which chromatin was reconstituted with pooled DNA and histone and either S phase or G_1 phase nonhistone chromosomal proteins, it was demonstrated that this difference in *in vitro* transcription of histone sequences is due to the histone chromosomal protein portion of chromatin.

In order to determine whether this is attributable to some component of the S phase chromosomal proteins that promotes the transcription of histone genes, a component of the G_1 phase chromosomal proteins that inhibits histone gene transcription, or both, chromatin

from both G_1 and S phase cells was dissociated and then reconstituted in the presence of additional chromosomal proteins. The results of this study confirm that it is the nonhistone chromosomal proteins that are responsible for the cell cycle stage specific differences in *in vitro* histone gene transcription and further show that these differences can be accounted for by a component or components of the S phase nonhistone chromosomal proteins that has the capacity, when reconstituted in the presence of G_1 chromatin, to render the histone genes transcribable in a dose dependent fashion.

To determine which component of the S phase nonhistone chromosomal proteins is responsible for the ability of these proteins to activate histone gene transcription, the S phase chromosomal proteins were fractionated using ion exchange and gel filtration chromatography. While the most purified fraction is not homogenous, it contains only 0.04-0.06% of the total chromosomal proteins but has sufficient ability to activate histone gene transcription to account for all the activity present in the total chromosomal proteins. This histone gene activator has an apparent molecular weight on Sephadex G-100 of 40,000-60,000 daltons, has a protein-like density in CsCl, is resistant to digestion with micrococcal nuclease, and is also able to activate histone gene transcription from chromatin of both quiescent WI-38 human diploid fibroblasts and mouse liver.

INTRODUCTION

A. Chromosomal Proteins and the Regulation of Gene Expression

1. Eucaryotic Genome

In order to understand the orderly sequence of changes in gene expression that occur during normal differentiation and function, as well as to understand abberations that may occur in these processes, it is necessary to understand the mechanisms by which specific eucaryotic genes are regulated. While our conception of gene regulation has been strongly influenced by work on procaryotic systems, particularly on phage lambda and the lac operator of E. coli, there are a number of features of the eucaryotic chromosome which are not adequately explained by these procaryotic models. The genome of eucaryotic cells is extremely large compared to that of bacteria, containing 100 to 1000 times more DNA. Approximately half of this DNA consists of repetitive sequences which are found in families of 10s, 100s or even 1000s of similar but nonidentical members scattered over the genome.[1] The function of these repetitive sequences is not known but they have been observed to be transcribed in a tissue specific fashion.[2-4] Even if one considers only DNA which is unique, or nearly so, a human cell contains approximately 500 times more DNA than E. coli. Since most of the known biosynthetic pathways are already represented in bacteria, a large portion of the DNA may be involved in regulation or may be

noninformational. Another major difference is that, unlike the genome of bacteria which consists primarily of DNA, the genome of a eucaryotic cell is a nucleoprotein complex referred to as chromatin which consists of approximately equal amounts of DNA and histone, and variable amounts of nonhistone chromosomal proteins. In the eucaryotic chromosome at any given time most of the genetic information is repressed.[2,5] This restricted capacity of the eucaryotic genome to function as a template for in vitro and in vivo DNA dependent RNA synthesis has been attributed to the histones or basic chromosomal proteins,[6-8] which are generally believed to act in a relatively nonspecific manner to repress large segments of the genome on a more or less permanent basis.[9] In contrast, the nonhistone chromosomal proteins have been implicated as having regulatory functions and may be responsible for the transcription of specific limited regions of the genome.[10-14] Although extreme caution must be exercised in drawing conclusions about gene regulation in mammalian cells based on results obtained from procaryotic systems, the concept which has evolved is that gene regulation in mammalian cells occurs by a mechanism similar to that proposed by Jacob and Monod[15] in 1961 with the E. coli lac operon. Specifically, it is generally believed that although the processes involved are undoubtedly somewhat more complex in a eucaryotic cell, the mammalian genome is repressed or derepressed by regulatory molecules acting on DNA and allowing or inhibiting its transcription.

Several elegant models for the control of transcription in higher organisms have been proposed. In their model, Britten and Davidson[16] postulate that the primary regulatory elements are a set

of activator RNAs. These RNAs are transcribed from what are called integrator genes under the control of sensor genes in response to specific inducer molecules such as hormones or metabolites. The activator RNAs bind in a sequence specific fashion to regions of the DNA called receptor genes. These are adjacent to structural genes whose transcription they activate. It should be noted that Britten and Davidson have pointed out that this model is logically equivalent to one in which the primary regulatory molecules are activator proteins coded for by the activator RNAs. A similar model has been proposed by Georgiev.[17] In this model the operon in higher organisms consists of an informational zone and a noninformational zone. Transcription is initiated by activator proteins which bind to acceptor loci adjacent to the promoter site in the noninformational zone. There can be more than one acceptor locus in each operon and different operons can contain identical acceptor loci. Thus one activator protein can initiate transcription of several operons each producing a different mRNA. After transcribing the noninformational zone containing the acceptor loci, the polymerase continues to transcribe the informational zone containing a collection of structural genes. After transcription, the giant RNA is degraded into smaller fragments and the structural gene transcripts are transferred to the cytoplasm. Since histones are known to inhibit RNA transcription, a number of models have dealt with mechanisms by which this repression can be lifted. It was initially suggested by Frenster[18] that in transcriptionally active chromatin that histones are displaced from the DNA by nuclear polyanions such as nonhistone chromosomal proteins. Based on the discovery that the nonhistone chromosomal proteins

are highly phosphorylated, Kleinsmith et al.[19] suggested that histones may be removed from a histone-DNA complex by the interaction of the negatively charged phosphate group of the nonhistone chromosomal proteins with the positively charged histones. The finding that histones stimulate the phosphorylation of the nonhistone chromosomal proteins led to a refinement of this hypothesis. According to the newer model[20] nonhistone proteins bind specifically to DNA in the presence of histone resulting in the phosphorylation of the nonhistone chromosomal proteins. The increased negative charge of the nonhistone chromosomal proteins strengthens the nonhistone chromosomal protein-histone attraction to a point that is sufficient to displace the histone from the DNA, thereby freeing the DNA template from histone repression and allowing transcription to take place. Modification of histones such as acetylation and phosphorylation may then "fix" regions of the chromatin in an "open" or transcribable conformation.[21] It is apparent at present, however, that very little is known about the mechanisms by which regulatory molecules interact with the genome to regulate the transcription of specific gene loci in eucaryotic cells.

2. Histones

Histones have been implicated by a number of investigators as having a very important role in the structure and regulation of the mammalian genome and thus in recent years a considerable amount of effort has been devoted to studying the biochemistry of these basic chromosomal proteins. The findings are summarized in a number of

reviews[22-25] as well as in several monographs.[26,27] Histones are positively charged proteins ranging in molecular weight from approximately 12,000 to 21,000 which are enriched in arginine and lysine residues and which completely lack tryptophan. They may be separated into five major classes by ion exchange chromotography,[28] polyacrylamide gel electrophoresis[29] and selective precipitation;[30] following the CIBA Foundation nomenclature[31] these are H_1 (very lysine rich), H_{2a} and H_{2b} (lysine rich), and H_3 and H_4 (arginine rich).

In nuclei or chromatin, the bulk of the DNA, in units of approximately 200 base pairs, is associated with bead-like nucleosomes on nu bodies.[32] These nu bodies can be visualized by electron microscopy[33] or detected by their resistance to nucleases.[34] The core of the nu bodies is thought to consist of two each of the histones H_{2a}, H_{2b}, H_3 and H_4.[32] Histone H_1, on the other hand, is thought to be associated with the outside of the nu bodies or with the DNA in the regions between the nu bodies.[35] The histones appear to be very important in the structure of these chromatin subunits since as has been shown by Sollner-Webb et al.,[36] these structures can be reconstituted with purified DNA and a mixture of the four histones H_{2a}, H_{2b}, H_3 and H_4.

The main argument for considering histones as gene regulators in mammalian cells comes from the demonstration that they inhibit in vitro DNA dependent RNA synthesis. Huang and Bonner[6] have shown that when DNA is complexed with increasing amounts of histone that there is a progressive decrease in the ability of the DNA to serve as a template

for the synthesis of RNA, with transcription being completely inhibited at a DNA to histone ratio of 1:1 (the ratio of histone to DNA generally found in the chromatin of intact cells). Also, Allfrey et al.[7] have shown that removal of histones from nuclei results in an increase in RNA transcription. Additional evidence that histones might be involved in the regulation of gene expression comes from the demonstration that they can be methylated,[37] acetylated,[38] and phosphorylated[39] independently of synthesis. These modifications can be correlated with changes in transcription associated with proliferation,[40-43] stimulation by hormones[44-46] and development,[47] as well as with chromosome condensation during mitosis.[48,49] As has been shown most clearly by Langan and Hohman[50] these modifications are associated with defined amino acid residues of histone fractions and are regulated by specific enzymes. These workers have demonstrated that in nonproliferating cells the serine 37 residue of histone H_1 is phosphorylated by a cyclic AMP dependent protein kinase. In proliferating cells, however, phosphorylation of histone H_1 involves sites other than serine 37, occurs to a large extent on threonine as well as serine residues and is catalyzed by an enzyme which is unaffected by cyclic nucleotides. The importance of histone modification in determining the structural and transcriptional properties of the genome is also shown by the fact that histone modifications alter DNA binding,[51,52] histone-histone relationships and alters the ability of histones to repress RNA synthesis.[51,53,54] The early enthusiasm produced by these results was soon tempered by a growing body of data which cast severe doubt on the ability of histones to function as specific gene repressors. John and Hoare[55]

have pointed out that in vitro determinations of DNA dependent RNA synthesis on a histone DNA complex may merely be an extremely sophisticated way of measuring precipitation since at least under in vitro conditions, histones precipitate DNA and thus make RNA polymerase and other transcribing factors inaccessible to the DNA template. Furthermore, even taking into account post-translational modifications, histones exhibit only a very limited species and tissue specificity. In fact, the amino acid sequence of histone H_4 calf thymus and pea seedling has been determined and has been shown to differ in only two amino acid residues.[56] It has also been shown that amounts of histones present in active and inactive tissues[57] as well as in euchromatin and heterochromatin are similar.[58,59] Thus, it is reasonable to conclude that while histones are undoubtedly intimately involved in the "packaging" of the genome and the repression of genetic information, they exhibit a uniformity and lack of specificity which makes it unlikely that they are the molecules which are primarily responsible for the specificity of gene readout.

3. Nonhistone Chromosomal Proteins

a. Correlative studies

Recently a considerable amount of attention has been focused on the nonhistone chromosomal proteins as possible regulators of gene expression in eucaryotic cells. This work is summarized in a number of recent reviews.[20,27,60-62] In contrast to the histones which are extremely stable proteins, the nonhistone chromosomal proteins exhibit

a spectrum of stabilities with half lives ranging from several minutes to several cell generations.[63] They are synthesized throughout the cell cycle, even during mitosis,[64,65] and their synthesis is generally unaffected by the inhibition of DNA replication.[66,67] One of the primary reasons for considering the nonhistone chromosomal proteins as gene regulators is that, unlike the histones, they are extremely heterogeneous.[20,60-62] These proteins give very complex patterns when analyzed by SDS gel electrophoresis and it has been shown by two dimensional systems that there are at least 500 different components.[68] This probably does not reflect true heterogeneity of these proteins, however, because each spot on these slab gels may contain several different components and because molecules which are present in less than 4000 copies per nucleus would probably not be detected by these procedures.[69]

Not only are the nonhistone proteins very heterogeneous, they also show a great deal specificity. Although the majority of the nonhistone proteins may be enzyme or structural components which are common to all cells[70,71] it has been shown that there are differences in nonhistones between different species,[72] different tissues,[20,60-62] and different cell types of the same tissue.[73-75] In addition, changes in the composition and metabolism of various molecular weight classes of nonhistone chromosomal proteins accompany modifications in gene expression associated with hormonal stimulation,[76,77] development,[78,79] differentiation,[80,81] stimulation of cell proliferation,[82-84], progression through the cell cycle,[85,86] activation of chromatin for transcription,[87,88] response to various pharmacological agents,[89] viral

infection and transformation,[90-93] and a broad range of other biological phenomenon. An additional source of heterogeneity for the nonhistone proteins is that they can be methylated,[94] acetylated[95] and phosphorylated[19,96-106] independently of synthesis.

The most extensive and best characterized of these post-translational modifications is phosphorylation. The nuclear proteins are approximately 0.1% alkali labile phosphorous by weight.[96] More than 90% of the nuclear protein bound phosphate is associated with the nonhistone chromosomal proteins, and a class of nonhistone proteins can be extracted which contains approximately 1.0% phosphate by weight.[96] The phosphorus is present primarily as phosphoserine which is present in sufficient quantities to account for five out of every hundred amino acids present. Nuclear proteins rapidly incorporate radioactive phosphate *in vivo* or *in vitro* and the nature of this phosphorylation reaction has been studied by Kleinsmith *et al.*[19] who incubated intact calf thymus nuclei *in vitro* with ^{32}P orthophosphate. The phosphorylation reaction is energy dependent since it is blocked by inhibitors of ATP synthesis such as iodoacetate and 2,4-dinitrophenol. There was no effect on the incorporation of phosphate into proteins when puromycin was used to inhibit protein synthesis up to 90% indicating that phosphate is attached to the protein after the polypeptide chain is synthesized. Pulse chase experiments with ^{32}P orthophosphate and ^{14}C serine show that 70-80% of the incorporated phosphate was lost from the proteins during a two hour chase while essentially no breakdown of protein occurred. The removal of phosphate groups therefore appears to be independent of protein degradation. Similar results which indicate that the nonhistone

proteins are continuously being phosphorylated and dephosphorylated at high rates within the nucleus have been obtained by several other investigators using *in vitro* systems involving nuclei or chromatin.[97-99] By means of polyacrylamide gel electrophoresis it has been shown that the phosphorylated nonhistone chromosomal protein fraction is highly heterogeneous and that although the overall patterns have many common features, there are differences between different tissues[99-101] and between the same tissue following stimulation by hormones,[102,103] stimulation to proliferate[104] or progression through the cell cycle.[105] Phosphorylation of the nonhistone proteins is catalyzed by a number of protein kinases some of which are cyclic AMP dependent.[106] These kinases have different substrate specificities and are tissue specific.[106]

b. Reconstitution as a specific probe of chromosomal protein function

The findings presented thus far suggest that the nonhistone chromosomal proteins exhibit a heterogeneity and specificity which is compatible with a significant role of these proteins in regulating the transcription of genetic information, but at best the evidence is circumstantial. Dissociation of chromatin into its constituent nucleic acid and protein components followed by reconstitution with selected classes of these macromolecules and assay of the RNAs transcribed from such reconstituted chromatin, however, has proved to be an extremely powerful approach which has provided direct evidence for the regulation of transcription by a nonhistone chromosomal protein.

Essentially, chromatin is reconstituted from isolated DNA, histones, and nonhistone chromosomal proteins by dissolving the components in 3.0 M NaCl, 5.0 M urea, 0.01 M Tris at pH 8.3 and then slowly removing the NaCl by gradient dialysis. Gilmour and Paul[107] demonstrated by RNA/DNA hybridization that the RNA transcribed by bacterial RNA polymerase from reconstituted calf thymus chromatin is indistinguishable from RNA transcribed from native chromatin, and that both were extensively similar to natural RNA from calf thymus. In other experiments, Bekhor et al.,[108] Gilmour and Paul,[109] Stein and Farber,[66] Stein et al.[110] and Spelsberg and Hnilica[111] have reconstituted chromatin using the high salt-urea method and have obtained evidence that the reconstituted chromatin is indistinguishable from the original chromatin based on both RNA/DNA hybridization and studies of template capacity. Further evidence supporting the fidelity of reconstitution comes from the demonstration that the thermal denaturation properties,[108] susceptibility to thymine base damage by gamma radiation,[112] and appearance in the electron microscope[113] are not altered in reconstituted chromatin. Stein et al.[110] have reported that the protein to DNA ratio, polyacrylamide gel electrophoretic profile in both SDS and urea gels, the binding of a reporter molecule with specificity for the minor groove of DNA, the number of sites available for the addition of nucleotides, and the circular dichroism spectrum of native and reconstituted chromatin are essentially identical.

One of the first uses of the technique of chromatin reconstitution to directly examine the role of the nonhistone proteins in regulating transcription was the work of Stein and Farber[66] who

investigated factors responsible for the differences in template capacity of mitotic and S phase chromatin. In these experiments chromatin from both mitotic and S phase cells was dissociated into DNA, histones, and nonhistones, and chromatin was then reconstituted with pooled DNA and histones and either S phase or mitotic nonhistone chromosomal proteins. These experiments demonstrated that the nonhistone chromosomal proteins determine the availability of DNA as a template for DNA synthesis since chromatin reconstituted in the presence of mitotic nonhistone proteins exhibited a lower capacity for RNA synthesis than chromatin reconstituted with S phase nonhistones. To eliminate the possibility that histones might be involved in these alterations in template capacity, reconstitution experiments were also performed in which the pooled DNA and nonhistone proteins were reconstituted either with S phase or mitotic histones. In this case, the transcriptional activities of the two types of reconstituted material were identical. In other studies using the technique of chromatin reconstitution, the nonhistone chromosomal proteins were shown to be responsible for the differences in template capacity of WI-38 fibroblast chromatin from contact inhibited cells and those that have been stimulated to proliferate.[114]

While these experiments indicate that the nonhistone chromosomal proteins play an important role in regulating the total amount of RNA transcribed, they give no information about the transcription of specific sequences. In an attempt to investigate the role of the nonhistone chromosomal proteins in the regulation of specific RNA sequences, Gilmour and Paul[109] analyzed the RNA transcribed from

reconstituted chromatin preparations by RNA/DNA hybridization. In these experiments chromatin from thymus and bone marrow was dissociated and fractionated and then chromatin was reconstituted with pooled DNA, pooled histone, and either thymus or bone marrow nonhistone chromosomal proteins. When pooled DNA and histone were reconstituted with nonhistones from bone marrow, the RNA transcribed from this chromatin was shown by competition hybridization analysis to resemble that normally transcribed from bone marrow chromatin. In contrast, when pooled DNA and histone were reconstituted with thymus nonhistone proteins the RNA synthesized from the reconstituted chromatin resembled that normally transcribed from thymus chromatin. Thus, the presence of tissue specific nonhistone proteins seems to determine the pattern of genes which will be transcribed in various tissues. In interpreting these results, however, it is very important to realize that the RNA/DNA hybridizations were carried out with total cellular DNA under conditions where only the moderately or highly reiterated sequences would hybridize.

c. Measurement of transcription of specific genes from chromatin

In order to extend the technique of reconstitution to the study of specific gene loci as well as to avoid some of the technical difficulties with RNA/DNA hybridization encountered in earlier studies one can utilize DNA molecules complementary in sequence to specific messenger RNAs as high resolution probes for the transcription of those particular genes. The use of complementary DNA probes to detect

specific RNA sequences does not require the RNAs to be translated or to bind to polysomes, the mRNA sequences can be detected if they are part of a larger molecule or even if they are partially degraded (although they are partially degraded this can be detected by a lowering of the melting temperature of the RNA/DNA hybrids), the assay is extremely specific, very small amount of RNA can be assayed, and very low concentrations of the specific RNA can be detected in the presence of high concentrations of many different RNAs.

Using a DNA probe complementary in sequence to mouse globin mRNA, Gilmour and Paul[115] have shown that globin mRNA sequences can be transcribed *in vitro* from chromatin of fetal liver (which is an erythropoetic organ) but not from the chromatin of mouse brain. Similar tissue specific transcription of globin sequences were observed by Axel *et al.*[116] who looked at transcription of globin sequences in duck reticulocytes and in adult liver (which is not an erythropoetic organ). These investigators found that globin sequences could be transcribed *in vitro* from reticulocyte chromatin but not from that of adult liver.

Coupling the technique of chromatin reconstitution and the use of complementary DNA probes to detect specific RNAs, it has been possible to provide direct evidence that the nonhistone chromosomal proteins are important in the regulation of globin gene transcription. Paul *et al.*[10] have shown that when brain chromatin (which does not transcribe globin sequences) is reconstituted in the presence of additional brain nonhistone chromosomal proteins, globin mRNA sequences

are not transcribed. When brain chromatin is reconstituted in the presence of fetal liver nonhistone chromosomal proteins, however, the globin genes are made available for transcription. The importance of nonhistone chromosomal proteins in the regulation of the transcription of globin genes was also shown by Barret et al.[11] In these experiments the erythropoetic tissue was chicken erythrocytes and the nonerythropoetic tissue was adult liver. Chromatin was reconstituted with the purified DNA and histones of erythrocytes and the nonhistone chromosomal proteins from either erythrocytes or adult liver. When chromatin was reconstituted with erythrocyte DNA, histone and nonhistone chromosomal proteins, it transcribed globin sequences and did so in a fashion identical to that of native erythrocyte chromatin. In contrast, chromatin reconstituted with erythrocyte DNA and histones and with adult liver nonhistone chromosomal proteins did not transcribe globin sequences. The importance of the nonhistone chromosomal proteins in the regulation of globin gene transcription is further supported by the studies of Chiu and coworkers.[12,117] In these studies using reticulocyte and brain chromatin, it was shown that a fraction of the nonhistone chromosomal proteins which represents less than 10% of the total proteins of reticulocyte chromatin is essential for the transcription of globin mRNA sequences from reconstituted chromatin preparations.

d. Fractionation of nonhistone chromosomal proteins

Fractionation of the nonhistone chromosomal proteins represents a formidable task. In addition to the heterogeneity of these

proteins--at least 500 classes of polypeptides[68]--major components of the nonhistone chromosomal proteins are insoluble under conditions employed for routine protein fractionation. Yet, in spite of these obstacles some progress has been made toward separating the nonhistone chromosomal proteins.

Analytical scale fractionation of the nonhistone chromosomal proteins has been successfully achieved by SDS-polyacrylamide gel electrophoresis. Recently a two-dimensional fractionation approach employing iso-electric focusing in one dimension and SDS-polyacrylamide gel electrophoresis in the second dimension has amplified the degree of resolution of this complex class of proteins.[68] A limitation of such procedures is that proteins prepared by these methods are not suitable for many biological assays--this being attributable in part to difficulties encountered in achieving complete renaturation and in completely removing SDS. However, nonhistone chromosomal protein fractions separated in SDS-polyacrylamide gels have been utilized to generate antibodies which exhibit specificity for native nonhistone chromosomal proteins.[118]

Fractionation of the nonhistone chromosomal proteins has been achieved on a preparative scale to a limited extent. Techniques which have been used include selective extraction of chromosomal protein fractions, ion exchange and gel filtration chromatography, and fractionation of the proteins on the basis of binding to nucleic acids. A major problem which has been encountered in purification of chromosomal proteins which are involved in the regulation of specific genes

has been that until very recently there has been no direct way to assay these molecules.

1. General considerations

In evaluating procedures for fractionation of the nonhistone chromosomal proteins, the same considerations which apply for isolation of nuclei and chromatin are operative, e.g., absence of cytoplasmic material and minimization of protease and nuclease activities. Since fractionation of chromosomal proteins frequently requires numerous steps and extensive periods of time, precautions to avoid degradation and irreversible denaturation are particularly important.[119] It should be emphasized that approaches to chromosomal protein fractionation must often be modified to accommodate differences inherent in various tissues and cell types.

A problem sometimes encountered in fractionating nonhistone chromosomal proteins is that fractions may contain nucleic acid--RNA as well as DNA. Such nucleic acids can complicate interpretation of experiments in which nonhistone chromosomal protein fractions are assayed in chromatin reconstitution systems for ability to render specific genes transcribable. If nucleic acids are covalently bound to the protein fraction, separation of the nucleic acids and chromosomal proteins is extremely difficult. However, separation of noncovalently associated nucleic acids and chromosomal patterns can be executed by buoyant density centrifugation in solutions of cesium salts and urea.

Buoyant density centrifugation in CsCl-urea has been used by Gilmour and Paul[120] to prepare chromosomal proteins under conditions that exclude endogenous RNA. Chromatin is dissolved in 55% CsCl, 4 M urea, 10 mM Tris (pH 8.0), 10 mM dithiothreitol, 10 mM EDTA at a concentration of 300 µg/ml. Four five-milliliter aliquots of the dissociated chromatin are then centrifuged at 40,000 rpm for 40 hours at 8°C in an MSE 10 x 10 titanium rotor or the equivalent (the remainder of the tube is filled with paraffin oil). The chromosomal proteins are found in the top 1.5 ml of the gradient, while the DNA and RNA form a pellet.

A procedure which has been used successfully by Modak and co-workers[121,122] to separate DNA, RNA and protein involves centrifugation in Cs_2SO_4 and urea. This method is particularly useful when the isolation of the nucleic acid components is desired. The sample is adjusted to 5 M urea, 0.54 mg/ml Cs_2SO_4, 30 mM NaCl, 20 mM Tris (pH 7.4), 5mM EDTA. Fifteen millimeter aliquots overlaid with paraffin oil are centrifuged for 50-62 hours at 170,000 g in a Beckman 60 Ti rotor at 22°C. RNA forms an unprecipitated band at density 1.71 g/cc, DNA bands at density 1.51 g/cc and protein floats.

2. Selective extraction of nonhistone chromosomal proteins

One approach to fractionation of nonhistone chromosomal proteins has been to extract classes of these molecules with various concentrations of salt and urea--the urea being used to facilitate extraction of tenaciously bound molecules and to maintain extracted

proteins in a soluble state. Serial extraction of rabbit liver chromatin with 5 M urea, 10 mM Tris (pH 8.0) and 5 M urea, 10 mM Tris (pH 8.0) containing increasing concentrations of NaCl, followed by high speed centrifugation after each extraction has been reported by Bekhor et al.[123] This procedure yields fractions which are extremely heterogeneous and which exhibit significant differences in their electrophoretic molecular weight profiles. Selective extraction of chromatin with salt and urea followed by high speed centrifugation has also been used by Chiu and coworkers in a procedure that utilizes differences in pH in the extraction steps.[124] In this procedure the majority of the nonhistone chromosomal proteins from rat liver are extracted by 5 M urea, 50 mM sodium phosphate (pH 7.5), the histones are extracted by 2.6 M NaCl, 5 M urea, 50 mM sodium succinate (pH 5.0), and the tightly bound nonhistone chromosomal proteins are extracted with 2.5 M NaCl, 5 M urea, 50 mM Tris (pH 8.0). Because both of these procedures involve repeated extraction of the chromatin followed by extensive centrifugation, they are time consuming and precautions must be taken to prevent proteolytic degradation.[119]

Nonhistone chromosomal protein fractions can also be obtained by extraction with NaCl in the absence of urea. One method that has been used by several workers is to extract nuclei or chromatin with 1.0 M NaCl.[125-127] This method has the advantage that when the salt concentration of the extract is lowered to 0.4 M, most of the DNA and histone as well as some of the nonhistone chromosomal proteins precipitate and can be removed by centrifugation. Another frequently

used procedure is to extract chromatin with 0.25 M or 0.35 M NaCl.[128-131] The proteins removed are electrophoretically similar to those that remain on the DNA,[128] show specificity of binding to DNA,[129] and have been implicated in Nicolini and coworkers as having an important role in determining the functional and structural changes that occur in chromatin of WI-38 cells which have been stimulated to proliferate.[130]

Extraction of nonhistone chromosomal proteins with phenol has proved to be an effective method for obtaining protein fractions, and details of the procedure have been reported.[100,132] By performing the phenol extraction at several pH's, additional fractionation can be achieved.[78] A note of caution is that extraction of nonhistone proteins with phenol is generally preceded by extraction of histones with dilute mineral acid. Both of these procedures may result in a certain amount of irreversible denaturation of the nonhistone proteins.

3. Nonhistone chromosomal protein fractionation procedures

Nonhistone chromosomal proteins can be isolated as a total class of macromolecules and then fractionated. Such procedures usually require separation of DNA from the total chromosomal proteins as an initial step, followed by fractionation of the total chromosomal proteins into histones and nonhistone chromosomal proteins. De-histonized chromatin (nonhistone chromosomal protein-DNA complexes prepared by removal of histones under mild conditions as described by Spelsberg and coworkers)[133,134] can also be used as a source of total nonhistone chromosomal proteins, and for immunological studies this material serves as an effective source of antigen.[135,136]

The method most commonly utilized for separation of DNA and chromosomal proteins involves dissociation of chromatin in 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.0) and centrifugation at 180,000 x G for 36 hours.[66,114] The chromosomal proteins are recovered from the supernatent fraction and contain less than 0.2% nucleic acid. DNA and chromosomal proteins can also be separated by dissociation of chromatin in 3 M NaCl, 5 M urea,10 mM Tris (pH 8.3) followed by molecular weight sieving on agarose.[137] The column is equilibrated in 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) and chromatography is carried out in the same buffer. Other techniques for separation of DNA from chromosomal proteins in dissociated chromatin include precipitation of nucleic acids with lanthanum chloride[138] and chromatography on hydroxylapatite.[139]

Ion-exchange chromatography is one of the methods which has been effectively employed for fractionation of nonhistone chromosomal proteins. The resins which have been used include DEAE-cellulose,[140,141] QAE-Sephadex,[109,142,143] SP-Sephadex,[144-146] and CM-Sephadex.[147,148] Chromatography is usually carried out in the presence of 5 M urea to maintain solubility of the proteins, and elution from these resins is generally achieved with a salt gradient.

DNA affinity chromatography is an approach which has been extensively utilized for fractionation of the nonhistone chromosomal proteins. DNA can be physically "trapped" in cellulose,[149] polyacrylamide,[150] agar,[151] or covalently bound to Sephadex,[129,152] or agarose.[129,153,154] The immobilized DNA can then be utilized to

select particular classes of nonhistone chromosomal proteins. Predicated on the assumption that components of the nonhistone chromosomal proteins have the ability to recognize and bind to defined genetic sequences, nonhistone chromosomal protein fractionation protocols often entail successive chromatography on heterologous and homologous DNA columns[155-157] and/or chromatography over DNA columns containing DNA of various Cr_0t values.[129,153] DNA binding has also been carried out in solution with DNA-protein complexes being isolated by sucrose gradient fractionation[100,158,159] or collection on nitrocellulose filters.[160] Although it is difficult to establish that the interactions observed between DNA and nonhistone chromosomal proteins in these procedures reflect the in vivo situation, nonetheless these affinity chromatography methods provide an effective method for fractionation of a complex and heterogenous group of macromolecules. It should also be noted that as defined DNA sequences become available these affinity chromatography procedures may be capable of yielding greatly increased resolution.

Other methods for nonhistone chromosomal protein fractionation on a preparative scale include molecular weight sieving using Sepharose,[147] Sephadex,[146,161] or the agarose[137] and polyacrylamide[161] Biogels, as well as chromatography on hydroxylapatite,[139] phosphocellulose[162] or calcium phosphate gel.[163]

4. Assessment of protein purification

In order to purify proteins responsible for a given activity one must not only have a way of fractionating proteins, but also a

way of assaying whether a given fraction has the activity. As shown in Table 1, there are a number of enzymatic activities which have been identified among the nonhistone chromosomal proteins which have formed the basis for purifying and characterizing the proteins involved. One of the major objectives of nonhistone protein fractionation, however, is to isolate classes and eventually individual molecular species which are involved in the regulation of specific genes. A number of investigators who have fractionated the chromosomal proteins have shown their fractions have different molecular weight profiles or have biological properties which might be expected for a regulatory molecule, such as species specificity,[144,145,164] the ability to bind to DNA,[155-159] or the ability to modify total RNA transcription.[165-167] A major problem that has been encountered is that there have been no direct assays for determining whether a given protein fraction is directly involved in the regulation of a specific gene. The reconstitution of chromatin with specific chromosomal protein fractions and assay of the RNA transcribed from such reconstituted chromatins with DNA probes complementary to specific RNAs offers such an assay.

B. Regulation of Histone Gene Expression During the Cell Cycle

The regulation of histone gene expression during the cell cycle offers an excellent opportunity to examine the regulation of specific genes in a situation where their expression is not a final differentiation event. The histones are synthesized only during the S phase of the cell cycle[168-171] and their synthesis is tightly coupled to that

Table 1

Enzymatic components of the nonhistone chromosomal proteins[60]

Enzyme components	Activity
DNA polymerases	polymerization of deoxyribonucleotides into DNA
RNA polymerases	polymerization of ribonucleotides into RNA
Nucleases	processing or degradation of RNA and/or DNA
Nucleotide ligases	joining of DNA segments during DNA replication and repair
Nucleotide exotransferases	addition of nucleotides to the ends of nucleic acids
Proteases	processing or degradation of proteins
Nucleic acid methylases	methylation of DNA or RNA
Protein methylases	methylation of histones and nonhistone chromosomal proteins
Protein kinases	phosphorylation of histones and nonhistone chromosomal proteins
Poly(adenosine diphosphate ribose) polymerase	addition of ADP-ribose moieties to chromosomal proteins
Poly(adenosine diphosphate ribose) glycohydrolase	removal of ADP-ribose moieties from chromosomal proteins
Protein acetylases	acetylation of histones and nonhistone chromosomal proteins
Protein deacetylases	removal of acetate groups from histone and nonhistone chromosomal proteins

of DNA.[169,172,173] During S phase, however, histones may account for as much as 10% of the total protein synthesized.[75]

The messenger RNAs which code for the histones have a size range of 7-12 S[174-176] and are found on polysomes of cells replicating their DNA[169,172,177,178] and in unfertilized eggs of sea urchins[179,180] and amphibians.[181] Their appearance on the polysomes of proliferating cells has been determined to depend on concomitant DNA synthesis.[13,172,174,182,183] The base composition of the histone mRNA is approximately 54% G+C,[178] placing them in a category between other messenger RNAs which are generally A+T rich and the ribosomal RNA which are more G+C rich. While the histone mRNAs contain both cap 1 and cap 2 structures on their 5' ends, in contrast to ribosomal RNA and many mRNAs, they lack internal methylated bases.[184] In HeLa cells they also lack the polyA sequence at their 3'OH end common to most messenger RNAs,[184,178] are rapidly transported to the polysomes after synthesis and possess a relatively short half life on the order of a few hours.[185-189]

Another unusual feature of the histone system is that while most of the DNA sequences which encode protein structures are unique, or nearly so, the histone genes are present in multiple copies. In sea urchins each histone gene has been determined to be 400-1200 fold reiterated.[190,191] In HeLa cells it has been estimated there are 10 to 20 copies of each histone gene per haploid genome.[192] Recently, a histone gene frequency of 80 copies has been estimated with human placental DNA.[193]

For the last several years, understanding the histone genes in HeLa S_3 cells has been a major thrust of this laboratory. The HeLa cell system was chosen for these studies for a number of reasons including their rapid and continuous proliferation, ease of growth and synchronization, and because of the large background of studies of histone messenger RNA metabolism in these cells. This cell line was originally derived from human cervical carcinoma tissue by Grey[194] and cloned and characterized by Puck.[195] The S_3 clone was first propagated by Puck and is now grown in suspension culture having a 19 hour cell cycle separated into the four phases as follows: 1 hour mitosis, 5 hour G_1, 9 hour S, and 4 hour G_2 phase.[196]

In order to directly detect histone messenger RNA sequences, a DNA complementary in sequence to histone messenger RNA has been synthesized.[174] Histone messenger RNA was isolated on the basis of its size and lack of polyA by repeated sucrose gradient centrifugation and chromatography on oligo dT cellulose. PolyA (average of 35 AMP residues) was added to the 3'OH termini by Dr. Rusty Mans in the Department of Biochemistry and Molecular Biology, University of Florida, with an ATP polynucleotidyl exotransferase from maize seedling.[197] This polyadenylated DNA was then transcribed with an RNA dependent DNA polymerase from AMV using dT_{10} as a primer. Using this cDNA probe it has been possible to directly show that the histone mRNAs are present on polysomes of S phase but not G_1 phase HeLa cells[198] and that they are absent from polysomes of S phase cells in which DNA synthesis has been blocked with cytosine arabinoside.[198]

In order to directly assay the availability of histone sequences for transcription we have transcribed chromatin from both S and G_1 phase HeLa cells and have assayed the RNA transcripts from the presence of histone sequences by hybridization with histone cDNA.[13,199] While the total amounts of RNA transcribed from G_1 and S phase cells is similar, histone sequences are found to be transcribed only from S phase chromatin.

The studies described in this thesis show that the difference in in vitro transcription of histone sequences from G_1 and S phase chromatin can be accounted for by a component or components of the S phase nonhistone chromosomal proteins which has the ability to activate histone gene transcription from G_1 phase chromatin in a dose dependent fashion. Using ion exchange and gel filtration chromatography this histone gene activator has been partially purified. The partially purified fraction contains only approximately 0.05% of the total S phase chromosomal proteins but has sufficient ability to activate histone gene transcription to account for all the activity present in total S phase chromosomal proteins. This histone gene activator has an apparent molecular weight on Sephadex G-100 of 40,000-60,000 daltons, has a protein-like density in CsCl, is resistant to micrococcal nuclease, and is also able to activate histone gene transcription from chromatin of quiescent WI-38 human diploid fibroblasts and from chromatin from mouse liver.

MATERIALS AND METHODS

A. Cell Culture

HeLa S_3 cells were maintained in exponential growth at a density of 2.0 x 10^5 to 6.0 x 10^5 cells/ml in Joklik-modified Eagles minimal essential media (Grand Island Biological Company), supplemented with 1.0 mM glutamine and 7% calf serum. Before the medium was supplemented, it was sterilized by Millipore filtration and stored at 4°C. The cultures exchange gases with the atmosphere in cotton plugged and magnetically stirred bottles in an environmentally controlled room at 37°C. Cell density was determined by the packed cell volume technique, in which 10 ml of culture was placed in 30 ml tubes with a 0.1 ml bottom stem and centrifuged 1300 x G in a PR-6 centrifuge (International Equipment Company) for three minutes. A packed cell volume of 0.01 ml represents 2.5 x 10^5 cells/ml in culture.

B. Cell Synchronization

1. Double Thymidine Block[200]

S phase cells were obtained by synchronization with two cycles of a 2 mM thymidine block. Exponentially growing cultures at 5 x 10^5 cells/ml were diluted to 3.75 x 10^5 cells/ml with warm supplemented

growth media and were brought to a concentration of 2 mM thymidine with 100 mM thymidine in Earle's balanced salt solution. After incubation for 12-16 hours, the cells were released from the first thymidine block by pelleting the cells at 1300 x G for 4 minutes in an International Model UV centrifuge at 37°C and resuspending the cells in warm supplemented growth media without thymidine. After 9 hours the cultures were again brought to a concentration of 2 mM thymidine and incubated for 12-16 hours. Cells were harvested 3 hours after release from the second thymidine block. Autoradiographic assessment of ^{3}H-thymidine labeled nuclei in culture that have been synchronized by this technique shows that approximately 98% of the cells are in S phase.[75]

2. Selective Detachment[201]

G_1 phase cells were obtained 1-3 hours after selective detachment of mitotic cells from semiconfluent monolayers. All procedures were carried out at 37°C. Partial synchronization was achieved by a single thymidine block for 12-16 hours. The cells were then released into warm supplemented Eagle's basal medium at a concentration of 5 x 10^5 cells/ml and 50 ml aliquots were transferred to 1 liter Blake bottles. After 7 hours, cells not adhering to the glass surface were removed by vigorous shaking and by decanting the media. The cell monolayers were then washed twice with nonsupplemented medium and 50 ml of supplemented Joklik modified Eagle's minimal essential medium was added. At 9-11 hours after plating began, mitotic cells were shaken into suspension and accumulated in 200 ml aliquots at 2 x 10^5 cells/ml.

The cells were then harvested after an additional hours of incubation at 37°C. Stein and Borun [75] have shown that when cells are synchronized by this procedure, incorporation of ^{3}H-thymidine into nuclei cannot be detected autoradiographically, reflecting the absence of S phase cells.

C. Isolation of Chromatin

The chromatin isolation procedure[75,202] was carried out at 4°C. Harvested cells were washed 3 times with 40 volumes of spinner salts (Grand Island Biological Company) to remove serum proteins. The cells were then lysed 3 times with 40 volumes of 80 mM NaCl, 20 mM EDTA, 1% triton X-100 (pH 7.2) by vortexing. This media causes hypotonic and detergent lysis of cells with fragmentation of the plasma membrane, the endoplasmic reticulum and the outer nuclear membrane.[75,202] The inner nuclear membrane may also have been partially or totally delipidated leaving a proteinaceous lamina layer as determined for other cell types.[203] Nuclei were pelleted by centrifugation at 1200 x G for 3 minutes and washed 2 times in 0.15 M NaCl, 10 mM Tris (pH 8.0) to remove the detergent. Nuclei isolated in this manner are free of cytoplasmic contamination when observed by phase contrast microscopy. Nuclei were lysed in 30 volumes of cold double distilled water by gentle homogenization with a wide clearance Dounce homogenizer. The chromatin was allowed to swell in an ice bath for 20 minutes and was then pelleted by centrifugation at 12,000 x G for 20 minutes in a Beckman JA-20 rotor at 4°C. Chromatin

of HeLa S_3 cells isolated by this procedure is composed of histones, nonhistone proteins, DNA and RNA in approximate ratios of 1.0:0.8 : 1.0:0.09.[23,27,66]

D. Transcription of Chromatin[13,199]

Chromatin was transcribed by using Fraction V E. coli RNA polymerase prepared according to the method of Berg et al.[204] Transcription was carried out for 60 minutes at 30°C in a Dounce homogenizer fitted with a wide clearance pestle and the reaction mixture was periodically homogenized to maintain chromatin solubility. The incubation mixture in a final volume of 3.5 ml contained: 40 mM Tris (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 20 µM EDTA, 0.008% 2-mercaptoethanol, 0.4 mM each of ATP, CTP, UTP, and GTP, 150 µg/ml DNA as chromatin, and 40 units of RNA polymerase (1 unit = 1 nanomole of ATP incorporation per 10 minute assay on 50 µG of calf thymus DNA). RNA was extracted as follows. At the end of the incubation, the reaction was brought to a concentration of 1% SDS, 0.1 M NaCl, 10 mM sodium acetate, 1.0 mM EDTA (pH 5.4), and incubated at 37°C for 15 minutes. The reaction mixture was extracted twice with equal volumes of phenol with subsequent addition of chloroform-isoamyl alcohol (24:1 volume/volume) and two more extractions were performed with equal volumes of chloroform-isoamyl alcohol alone. The nucleic acids were precipitated with 2 volumes of ethanol at -20°C. The precipitate was collected by centrifugation at 12,000 x G for 30 minutes and resuspended in 10 mM Tris, 0.1 M NaCl, 5.0 mM $MgCl_2$ (pH 7.4) containing 40 µg/ml

deoxyribonuclease I (ribonuclease free--Sigma) and incubated at 37°C for 60 minutes. Following one extraction with phenol chloroform-isoamyl alcohol and two with chloroform-isoamyl alcohol the aqueous phase containing the RNA transcripts was chromatographed on Sephadex G-50 fine and eluted with 10 mM sodium acetate, 0.1 m NaCl, 1.0 mM EDTA (pH 5.4). The RNA transcripts (which are found in the void volume) were precipitated with two volumes of ethanol at -20°C. For hybridization analysis the RNA was resuspended in 100 µl of 25 mM HEPES, 0.5 M NaCl, 10 M EDTA (pH 7.0).

E. Detection of Histone mRNA Sequences

1. Preparation and Properties of Histone cDNA

To detect the presence of histone-specific RNA sequences a single strand DNA complementary to histone mRNAs was synthesized using RNA-dependent DNA polymerase as described previously.[174] The 7-11 RNAs were isolated from polysomes of S phase HeLa cells by repeated sucrose gradient centrifugation and polyA containing RNAs were removed by oligo (dT) cellulose chromatography. The remaining 7-11 S RNAs directed only the synthesis of all five classes of histones in a cell-free protein synthesizing system from wheat germ although histone H_1 is synthesized in lower amounts than the other histones.[174] This mRNA preparation has also been shown to lack internal methylated ribonucleotides, indicating the absence of ribosomal RNA.[184] Poly A (an average of 35 AMP residues) was added to the 3'-OH termini of the histone mRNAs with an ATP-polynucleotidyl-exotransferase isolated

from maize seedlings. The isolation and properties of this enzyme have been reported.[197] The polyadenylated RNAs were then transcribed with RNA-dependent DNA polymerase from avian myeloblastosis virus using dT_{10} as a primer. The mean sedimentation coefficient of the cDNA in alkaline sucrose was 6.1 S which corresponds to an average size of approximately 400 nucleotides.[205,206] When the cDNA is annealed to the histone mRNA preparation isolated from the polysomes of S phase HeLa cells at 52°C in the presence of 50% formamide, the reaction proceeds with a $Cr_0t_{\frac{1}{2}}$ of 1.7×10^{-2} to a maximum of 63% hybridization as estimated by a double reciprocal plot.[207] Since the molar concentration of the nucleotide sequences in solution determines the rate of hybridization, comparison of the $Cr_0t_{\frac{1}{2}}$ of the histone mRNA to that of a kinetic standard such as globin mRNA (complexity of 1200 bases[208] and $Cr_0t_{\frac{1}{2}}$ in similar conditions of 3.8×10^{-3})[209] yields a calculated sequence complexity of approximately 5400 which is two times greater than expected for the total complexity of the five histone messages. This is, however, within the range of variation found for the rate of RNA-DNA hybridization.[207,210-212] When cDNA and histone mRNA are hybridized in the absence of formamide at 75°C, the $Cr_0t_{\frac{1}{2}}$ is 5×10^{-3}. From the $Cr_0t_{\frac{1}{2}}$ of the globin mRNA in these conditions ($Cr_0t_{\frac{1}{2}} = 2.0 \times 10^{-3}$)[11,213] the sequence complexity of the histone mRNA can be estimated to be 3000 bases. When the probe is annealed with E. coli RNA in either of the above conditions, no significant level of hybrid formation is detected. The low level of S_1 nuclease-resistant, TCA precipitable radioactivity may be accounted for by a limited amount

(3% of ^{3}H-cDNA which is not digested by S_1 nuclease in the incubation conditions used. When the hybrids are formed at 52°C in the presence of 50% formamide, thermal denaturation curves of the histone mRNA-cDNA hybrids exhibit a single transition with a T_m of 67°C in 50% formamide-0.5 m NaCl, 25 mM HEPES (pH 7.0), 1 mM EDTA. This T_m value is consistent with the reported base composition of histone mRNA of 54% G+C.[178]

Additional evidence for specificity of the histone cDNA is its ability to form hybrids with total polysomal RNA isolated from intact S phase HeLa cells ($Cr_o t_{\frac{1}{2}}$ = 1.8) and its lack of hybrid formation with G_1 polysomal RNA.[198] From these findings it is reasonable to conclude that ribosomal (5S, 18S, and 28S) and transfer RNA complementary sequences are not present in the histone cDNA. Furthermore, the polysomal RNA isolated from HeLa cells in which histone and DNA synthesis have been blocked by cytosine arabinoside does not form hybrids with the histone cDNA.[13] These results are consistent with data from several laboratories[172,174,182,183] which indicate that histone mRNA is not present on the polyribosomes of HeLa cells treated with inhibitors of DNA synthesis, and additionally rule out the possibility that the cDNA contains detectable amounts of sequences complementary to other S phase specific, nonpolyadenylated RNAs which have been reported to be insensitive to cytosine arabinoside.[189] We cannot exclude the possibility that our cDNA preparation contains material other than histone sequences, particularly if the RNA from which this material was reverse transcribed does not translate in a cell free

system. If such material is present, however, it has all of the properties discussed above that one would expect for histone sequences and, in addition, its transcription from chromatin is apparently regulated by the nonhistone chromosomal proteins in the same fashion as the histone sequences.

2. Hybridization Analysis

^{3}H histone cDNA and in vitro transcripts were hybridized in conditions of RNA excess at 52°C in sealed glass capillary tubes containing in a volume of 15 µl:50% formamide, 0.5 m NaCl, 25 mM HEPES (pH 7.0), 1 mM EDTA, 0.04 ng cDNA (27,000 dpm/ng) and various amounts of RNA. E. coli RNA was included in the reaction mixtures when necessary so that the final amount of RNA was 3.5 µg. After various incubation times, the reaction mixtures were assayed for hybrid formation using Fraction IV single-strand specific S_1 nuclease isolated from Aspergillus oryzae.[214] Each sample was incubated for 20 minutes in 2.0 ml of 30 mM sodium acetate, 0.3 M NaCl, 1 mM $ZnSO_4$, 5% glycerol (pH 4.6) containing S_1 nuclease at a concentration sufficient to degrade at least 95% of the single-stranded nucleic acids present. Acid precipitable radioactivity was determined by adding 4 ml 15% cold trichloroacetic acid (TCA) and collecting the precipitate on 0.45 micron HA nitrocellulose filters. The filters were washed with 50 ml of cold 10% TCA, dissolved in 1 ml of ethylene glycol monoethyl ether (cellusolve), and counted in toluene-base scintillation fluid containing 26% (V/V) cellusolve, 4.23 grams/l of 2,5-diphenyloxazole (PPO) and 52 mg/l

of 1,4-di (2-(5 phenyloxazolyl)) benzene (POPOP)(liquiflour-New England Nuclear). This scintillation fluid will be referred to as cellusolve-toluene.

3. Determination of Melting Temperature of Histone cDNA-RNA Hybrids

Melting temperature (T_m) determinations were made in the same buffer used for hybridization. In order to determine the melting temperature of the RNA-DNA hybrids, histone cDNA and RNA transcripts were hybridized at 52°C to a Cr_0t at which 80% of maximal hybridization was observed using a concentration of transcripts such that this required approximately 10 hours. Individual samples were then heated for 10 minutes at various temperatures and quick cooled in an ice-water slurry. The amount of hybrid remaining after incubation at each temperature was assayed with S_1 nuclease as described above.

F. Fractionation of Chromatin

1. Isolation of Histone and Nonhistone Chromosomal Proteins

To prepare total chromosomal proteins chromatin was first dissociated in 3.0 M NaCl, 5 M urea (pH 8.3) at a DNA concentration of 0.5 mg/ml. Solid NaCl and urea were powdered with a mortar and pestle and added to the chromatin pellet along with a small volume of 10 mM Tris (pH 8.3) at 4°C. After vortexing briefly to distribute the salt and urea throughout the chromatin pellet, this mixture was

diluted with 10 mM Tris (pH 8.3) to make the final concentration of salt and urea 3 M and 5 M, respectively. The mixture was then vortexed vigorously with intermittent cooling in an ice-water slurry to dissolve the salt and urea and dissociate the chromatin.

In order to prepare chromosomal proteins, nucleic acid was removed from the dissociated chromatin by centrifugation at 50,000 rpm for 50 hours in a Beckman 60 Ti rotor at 4°C. The total chromosomal proteins were then dialyzed against 4, 5 hour changes of 10 volumes of 5 M urea, 10 mM Tris (pH 8.3) at 4°C and histone and nonhistone proteins were separated by the QAE-Sephadex method of Gilmour and Paul.[109] In this procedure QAE-Sephadex A-50 (Pharmacia) previously equilibrated with 5 M urea, 19 mM Tris (pH 8.3) was added to the protein solution (1 gram of Sephadex per 25 ml of protein solution) and the slurry was mixed for 20 minutes at 4°C. The histones and approximately 10% of the nonhistone chromosomal proteins do not bind to the Sephadex and are collected by filtration. The nonhistone chromosomal proteins are then eluted from the QAE-Sephadex with 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3).

2. DNA Isolation

DNA was isolated by a modification of the Marmur[215] procedure with subsequent treatment with RNAase, pronase and phenol.[216] Cells were washed with Earle's balanced salt solution, suspended in 1.5% SDS at 60°C, and shaken for 20 minutes. After cooling, the suspension was brought to 1.0 M $NaClO_4$ and deproteinized by repeated extractions

with equal volumes of chloroform-isoamyl alcohol (24:1, V/V) by mixing for 20 minutes, centrifuging, and discarding the organic layer and interface. When interfacial material was no longer present, the aqueous phase was poured through 2 volumes of cold ethanol. The mixture was gently swirled and the precipitated DNA was spooled onto a glass rod. DNA was resuspended in 15 mM sodium citrate (pH 7.0), 0.15 M NaCl (SSC). Heat-treated bovine pancreatic ribonuclease A (Sigma) was added at a concentration of 50 mg/ml and the mixture was incubated for 30 minutes at 37°C. Self-digested pronase (Calbiochem) was added to 50 mg/ml and was incubated 1 hour at 37°C. The mixture was then extracted with SSC buffered phenol and chloroform-isoamyl alcohol. Extraction was repeated until interfacial material was absent. The DNA was precipitated with two volumes of ethanol, resuspended in 0.1 SSC, chilled, made 0.33 M sodium acetate, 10 mM EDTA and precipitated with 0.54 volumes of cold isopropyl alcohol.

G. Reconstitution of Chromatin

Chromatin was reconstituted at a DNA concentration of 0.25 mg/ml by an adaptation of the gradient dialysis procedure of Bekhor et al.[108] The chromatin components were mixed in 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) at 4°C and were dialyzed for four hours each against 20 volumes of 5 M urea, 10 mM Tris (pH 8.3) containing successively 1.5 M, 1.0 M, 0.8 M, 0.6 M, 0.4 M, 0.3 M, 0.2 M and 0.1 M NaCl. The preparation was then dialyzed for two changes against 5 M urea, 10 mM Tris (pH 8.3), without NaCl. The chromatin was recovered by centrifugation at

30,000 x G for 60 minutes. The gellatinous chromatin pellet was resuspended in water, allowed to swell at 0°C for 30 minutes and centrifuged at 30,000 x G for 30 minutes. The reconstituted chromatin recovered contained approximately 75% of the input DNA and had the protein-DNA ratio of native chromatin (1.8-2.0) over the range of protein to DNA input used in these studies.

H. Fractionation of S Phase Chromosomal Proteins

1. Labeling and Extraction of Total Chromosomal Proteins

The cells used in these studies were labeled for two generations during exponential growth and during all stages of a double thymidine block with 0.2 μCi/ml of 4,5-^{3}H L-leucine (Schwarz-Mann 61 Ci/mMole). To facilitate labeling the Joklik-Modified Eagles minimal essential media used for both exponential growth and for the synchronization contained only 1/10 of the normal concentration of the L-leucine. Chromatin was then isolated from the labeled S phase cells and was dissociated with 3 M NaCl, 5 M urea (pH 8.3) as described above. In order to remove nucleic acids, the dissociated chromatin was centrifuged for 40 hours at 50,000 rpm in a Beckman 60 Ti rotor at 4°C. Total S phase chromosomal proteins prepared by this procedure contained 0.2% or less nucleic acid as estimated by the ratio of absorbance at 280 and 260 nanometers and have a specific activity of 1.0 - 1.1 x 10^6 cpm/mg protein when counted in 3 M NaCl, 5 M urea, 10 mM Tris in a toluene-based scintillation fluid containing 33%

(V/V) triton X-100 (Palmetto Chemicals), 4.42 g/l of PPO and 55 mg/l POPOP (liquiflour-New England Nuclear). This scintillation fluid will be referred to as triton-toluene.

2. Fractionation on QAE-Sephadex

The QAE-Sephadex procedure used to fractionate the S phase nonhistone chromosomal proteins is an adaptation of the method of Gilmour and Paul.[109] Total S phase chromosomal proteins from which nucleic acid had been removed by ultracentrifugation was dialyzed against 4 changes for 5 hours each of 10 volumes of 5 M urea, 10 mM Tris (pH 8.3) at 4°C. The protein was then loaded on a column of QAE-Sephadex A-25 (which has been equilibrated with the same buffer) at a flow rate of 15-30 ml/cm^2/hr, using approximately 1 gram of Sephadex per 10 mg of protein. The proteins were then eluted at the same flow rate with 2 column volumes each of 5 M urea, 10 mM Tris (pH 8.3), containing 0.1, 0.25, 0.5 and 3.0 M NaCl. The eluent from the column was monitored by counting 100 µl of each fraction in 5 ml of triton-toluene scintillation fluid and the material eluting at each salt concentration was pooled. The total recovery of protein from the column is 50-75%.

The chromosomal protein fractions may be stored at this stage at either -20°C or in liquid N_2 after adjusting to 3.0 M NaCl by the addition of solid NaCl. At -20°C the solution does not freeze and there is no decrease in histone gene activator activity upon storage for several weeks; however, under these conditions the salt and urea

slowly crystallize. No decrease in activity has been noted even after several months of storage in liquid N_2.

3. Fractionation on SP-Sephadex

The protein eluted from the QAE-Sephadex column with 0.5 M NaCl was further fractionated with the strong cation exchanger SP-Sephadex (Pharmacia). The proteins were titrated to pH 5.2 with 1 M sodium acetate (pH 4.5) and were dialyzed for 5 hours each against 4 changes of 10 volumes of 5 M urea, 0.1 M NaCl, 0.2 M sodium acetate (pH 5.2). The proteins were then loaded on a column of SP-Sephadex C-25 (which had been equilibrated with the same buffer) at a flow rate of 2 column volumes per hour using approximately 2 grams of Sephadex per mg of protein. The proteins were then eluted at the same flow rate with 2 column volumes each of 5 M urea, 0.2 M sodium acetate (pH 5.2) containing 0.2 M and 0.4 M NaCl and then the 2 column volumes of 5 M urea, 3 M NaCl, 100 mM Tris (pH 8.3). The total recovery of protein from the column was approximately 50%.

It was subsequently discovered using chromosomal proteins from unsynchronized cells that comparable fractionation can be obtained with less SP-Sephadex (1 gram Sephadex per 3 mg protein) even when the proteins to be fractionated are in a large volume (20 column volumes). Since the bound proteins can be eluted from the SP column in a small volume this represents a very effective way of concentrating these proteins.

4. Chromatography on Sephadex G-100

Sephadex G-100 (40-120 micron) was swollen for 3 days at room temperature in 5 M urea, 3 M NaCl, 10 mM Tris (pH 8.3) containing 0.02% sodium azide. The Sephadex was then equilibrated to 4°C and a 1.5 x 27 cm column was packed. This column was allowed to stabilize by flowing for 24 hours with a hydrostatic head of 30 cm (approximately 15 ml/hr).

The proteins eluted from the SP-Sephadex column with 0.4 M NaCl were titrated to pH 8.3 with 1 M Tris base and were made 3 M NaCl by the addition of solid NaCl. One milliliter of the SP fraction, containing approximately 3.4 μg of protein, was mixed with 1 ml of 3M NaCl, 5 M urea, 10 mM Tris (pH 8.3) and was chromatographed on the Sephadex G-100 column with a hydrostatic head of 30 cm (a flow rate of approximately 15 ml/hr) collecting 2 ml fractions. The elution of protein from the column was monitored by counting 0.5 ml of each fraction in 20 ml of triton-toluene scintillation fluid. The total recovery of protein was greater than 90%.

I. Characterization of Chromosomal Proteins

1. Electrophoresis

Two different types of polyacrylamide gel electrophoresis were used in these studies. For characterization of chromosomal fractions obtained with QAE-Sephadex, 7.5% SDS polyacrylamide tube gels were used.[217] SDS polyacrylamide slab gels prepared by the method

of Laemili[218] were found to have much greater resolution, however, and were used for the subsequent work.

a. SDS tube gels

The SDS tube gel procedure was based on that of Bhorjee and Pederson.[217] The resolving gels were prepared with 7.5% (W/V) acrylamide (Eastman) and 0.28% (W/V) N,N-bisacrylamide (Eastman) as the cross linker in 0.1 M $NaPO_4$ (pH 7.0), 0.5 M urea, 5 mM EDTA, 0.1% SDS. The gels were polymerized with 0.05% N,N,N',N'-tetramethylethylenediamine (TEMED) (Eastman) and 0.1% ammonium persulfate. Immediately after the TEMED and ammonium persulfate were added, 0.6 x 7.5 cm gels were cast, overlaid gently with 5 mm of 0.1% SDS, 5 mM EDTA, 0.1% ammonium persulfate, and were allowed to polymerize. Complete polymerization required approximately 30 minutes. The stacking gels were prepared with 2.5% acrylamide and 0.09% N,N-methylene bisacrylamide as the cross linker in 0.01 M $NaPO_4$ (pH 6.0), 0.5 M urea, 5 mM EDTA, 0.1% SDS. After the addition of 0.05% TEMED and 0.08% ammonium persulfate, 2 cm stacking gels were cast on top of the resolving gel.

Proteins were prepared for electrophoresis by heating for 30 minutes at 60°C in 1% SDS, 1% 2-mercaptoethanol, 0.1 M $NaPO_4$ (pH 7.0) and were dialyzed against 100 volumes of 0.1% SDS, 0.1% 2-mercaptoethanol, 0.01 M $NaPO_4$ (pH 7.0). Twenty to fifty microgram aliquots were electrophoresed for 7 hours at 8 milliamps/gel using 0.1 M phosphate buffer (pH 7.0), 5 mM EDTA, 0.1 SDS as the running buffer. The gels were fixed overnight in 12% TCA, 40% ethanol, 7% acetic acid, stained

for 5 hours at 37°C in 0.25% Coomassie Brilliant Blue in 40% ethanol, 7% acetic acid and diffusion destained in 10% ethanol, 7% acetic acid. The gels were then scanned at 600 nm in a Beckman Acta II spectrophotometer equipped with a linear transport on a scale of 0-3 absorbance units.

b. SDS polyacrylamide slab gels

This SDS slab gel procedure is based on that of Laemili.[218] The resolving gel was prepared with 8.75% (W/V) acrylamide and 0.23% (W/V) in N,N-bioacrylamide as the cross linker in 0.375 M Tris (pH 8.8), 0.1% SDS. The gel was polymerized with 0.025% TEMED and 0.04% ammonium persulfate. Immediately after the catalysts were added a 14 by 10 cm slab 1.5 mm thick was cast, overlaid gently with 0.1% SDS and allowed to polymerize. Polymerization required approximately 45 minutes. After the resolving gel had polymerized a 1 cm stacking gel was cast. The stacking gel was prepared with 3% acrylamide and 0.08% N,N methylene bisacrylamide in 0.125 M Tris (pH 6.8), 0.1% SDS. The stacking gel was polymerized with 0.1% TEMED and 0.03% ammonium persulfate.

Samples were prepared for electrophoresis by heating at 60°C for 30 minutes in 1% SDS, 1% 2-mercaptoethanol and were then dialyzed against 100 volumes of 0.0625 M Tris (pH 6.8), 0.1% SDS, 0.1% 2-mercaptoethanol. The proteins were made 5% in glycerol and 20-50 µg aliquots were electrophoresed for approximately 2 hours at 30 milliamps per slab using an electrophoresis buffer which contained

0.025 M. Tris base, 0.19 M glycine and 0.1% SDS. After electrophoresis the gels were fixed overnight with 12.5% TCA, 40% ethanol, 7% acetic acid and were then placed in 10% ethanol, 7% acetic acid for 12 hours. The gels were stained for 5 hours in 0.25% Coomassie Brilliant Blue in 40% ethanol, 7% acetic acid and diffusion destained in 10% ethanol, 7% acetic acid. The gels were then photographed with Kodak high contrast copy film (ASA 64) and printed on polycontrast paper using a #4 filter.

c. Concentration of proteins for electrophoresis

In order to concentrate proteins which are present in high concentrations of urea and salt for electrophoresis, the proteins were dialyzed for 2, 2 hour changes against 100 volumes of 1.0 M urea, 0.5 M NaCl, 10 mM Tris (pH 8.3) and 0.3 volumes of 100% TCA was then added. This mixture was incubated at 0°C for 30 minutes and precipitate was collected by centrifugation at 8000 x G for 8 minutes in an Eppendorf 3200 centrifuge. The precipitated protein was then washed twice with cold acetone to remove the TCA and was dissolved in SDS slab gel buffer (0.0625 M Tris-HCl (pH 6.8), 0.1% SDS, 0.1% 2-mercaptoethanol) by boiling for 2 minutes. Using this technique it was possible to obtain reproducible SDS slab gel electrophoretic profiles on as little as 20 µg of chromosomal proteins present in an initial volume of 1 ml of 3 M NaCl, 5 M urea.

2. Molecular Weight Determination

In order to determine the approximate molecular weight of histone gene activator activity in total chromosomal proteins, S phase total chromosomal proteins were chromatographed on a calibrated column of Sephadex G-100 in 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) at 4°C. Sephadex G-100 was swollen for 3 days at room temperature in 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3), 0.02% sodium azide and was then cooled to 4° and a 1.5 x 28 cm column was packed using a hydrostatic head of 30 cm. After packing, the column was allowed to flow for 24 hours with a hydrostatic head of 30 cm (a flow rate of approximately 15 ml per hour) to stabilize. This column was calibrated by chromatographing 2 ml of a 2 mg/ml solution of Blue Dextran 2000 (Pharmacea) to determine the void volume and by chromatographing 2 ml of 2 mg/ml solutions of bovine serum albumin, ovalbumin and whale skeletal muscle myoglobin (Sigma). The elution of the dextran and proteins was monitored by their adsorption at 620 and 280 nm, respectively, and their elution volume was measured as the volume of buffer eluted from the beginning of the load to the midpoint of absorbance peak. The elution constant (K_{av}) of the proteins was calculated from the relationship:

$$K_{AV} = \frac{V_E - V_0}{V_T - V_0}$$

where V_E is the elution volume, V_0 is void volume and V_T is the total volume of the G-100 bed. Since there is approximately a linear

relationship between K_{AV} of Sephadex G-100 and the logarithm of molecular weight for many different proteins between 4,000 and 100,000 daltons, these data can be used to construct a calibration curve.

After the column had been calibrated, 2 ml of 0.5 mg/ml S phase total chromosomal proteins in 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) was chromatographed under the same conditions used for calibration. The fractionated protein was pooled into 4 fractions and the range of elution volume included in each fraction was measured. From the elution volumes, the K_{AV} and thus the range of apparent molecular weight in each fraction can be calculated. In order to assay the fractions for their ability to activate histone gene transcription, 100 µg of each fraction was reconstituted in the presence of 1 mg of G_1 DNA as chromatin, the reconstituted chromatins were then transcribed, and the purified transcripts were annealed with histone complementary DNA.

When Sephadex G-100 was used to fractionate the 0.04 M SP-Sephadex fraction, the same calibration procedure was used to calculate the apparent molecular weight of the Sephadex G-100 fractions.

3. Binding of Chromosomal Protein Fraction to Chromatin During Reconstitution

To determine how much of the 0.4 M SP-Sephadex fraction binds to chromatin during chromatin reconstitution, unlabeled G_1 chromatin was reconstituted in the presence of ^{3}H-leucine labeled SP-Sephadex fraction. G_1 phase chromatin was dissociated in 3 M NaCl, 5 M urea (pH 8.3) and then 3 mg of this chromatin (containing approximately 1 mg of DNA) was mixed with 8 µg of the tritium labeled 0.4 M SP-Sephadex

fraction (1 - 1.1 x 10^6 cpm/mg). Half of this mixture was immediately adjusted to 1% SDS, 1% 2-mercaptoethanol, 10 mM sodium phosphate (pH 7.0), was boiled for 5 minutes and was frozen. The other half was reconstituted as described above at a DNA concentration of 0.25 mg/ml. After reconstitution the chromatin was centrifuged at 30,000 x G for 1 hour. The reconstituted chromatin was resuspended in 1 ml of water and was made 1% SDS, 1% 2-mercaptoethanol, 10 mM sodium phosphate (pH 7.0) and boiled for 5 minutes.

To determine how much label was in each fraction, 100 µl aliquots were counted in 5 ml of triton-toluene scintillation fluid. The amount of DNA present in each fraction was estimated by their absorbance at 260 nm.

The fractions were analyzed by SDS slab gel electrophoresis, as described above, using a 3 mm thick slab with 4 cm wide wells. After being stained and photographed, the gel was cut into 3 mm wide slices by hand with a stainless steel blade. The slices were then dehydrated for 48 hours at 37°C, were solubilized with 1 ml of 30% H_2O_2, and were counted in 20 ml of triton-toluene scintillation fluid.

4. Ultraviolet Spectrum

The ultraviolet absorbance spectrum of the G-100 fraction of log phase chromosomal proteins was measured using a Beckman Acta II double beam spectrophotometer with 1 cm quartz cuvettes. The reference cuvette contained 3 M NaCl, 5 M urea and 10 mM Tris (pH 8.3).

5. Centrifugation in CsCl

a. Removal of nucleic acids from total chromosomal proteins

To remove residual nucleic acids from S phase total chromosomal proteins, they are dialyzed at a concentration of 0.6 mg/ml against 2 changes of 50 volumes of 5 M urea, 10 mM Tris (pH 8.3) and then made 0.41 mg/ml in CsCl. Five milliliters of this solution was centrifuged for 48 hours at 35,000 rpm at 4°C in a Beckman SW 50.1 rotor. The chromosomal proteins were found in the top half of the gradient and nucleic acids pelleted. When a mixture of 0.5 mg of ^{3}H-uridine labeled RNA and ^{3}H-thymidine labeled DNA and 2.5 mg of total chromosomal proteins was fractionated by this procedure, greater than 99.5% of the labeled nucleic acid was removed from the protein fraction.

b. Density of histone gene activator in CsCl

In this study, the 0.4 M SP-Sephadex fraction was banded in a CsCl density gradient and the distribution of the histone gene activator activity and the protein in the gradient were compared. Approximately 2.6 µg of ^{3}H-leucine labeled S phase 0.4 M SP-Sephadex fraction (1.0 x 10^{6} cpm/mg) was mixed with 500 µg of G_1 phase total chromosomal proteins as a carrier and was dialyzed for 12 hours against 100 volumes of 5 M urea, 10 mM Tris (pH 8.3). The protein solution was made 0.412 mg/ml in CsCl (Grand Island Biological Company) and was adjusted to 5 ml with 5 M urea, 10 mM Tris (pH 8.3). This mixture was centrifuged

for 80 hours at 37,500 rpm at 4°C in a Beckman SW 50.1 rotor. After centrifugation the gradient was fractionated with an ISCO model 640 gradient fractionator by lifting the gradient with flourinert (Instrumentation Specialties Company) and 0.6 ml fractions were collected. The density of the fractions was determined by cooling them to 4°C and then weighing 500 µl aliquots. The distribution of ^{3}H-leucine in the gradient was measured by counting 100 µl of each fraction in 5 ml of triton-toluene scintillation fluid. Fractions 1 and 2, 3-5, and 6-8 were pooled and 100 µl of each pooled fraction was assayed for its ability to activate histone gene transcription by reconstitution in the presence of 1 mg of G_1 DNA as chromatin.

6. Digestion with Micrococcal Nuclease

a. Activity of micrococcal nuclease in urea

In order to measure the activity of micrococcal nuclease in urea, the enzyme was incubated with 18S ribosomal RNA and both single and double stranded calf thymus DNA. HeLa 18S ribosomal RNA was prepared in this laboratory by Alex Litchler by extracting polysomes with phenol and chloroform-isoamyl alcohol and then separating the RNA by centrifugation through a sucrose density gradient. Calf thymus DNA (Sigma) was sonicated to reduce its viscosity and was denatured by heating for 5 minutes at 100°C and quick cooling in an ice-water slurry. The nucleic acids, at a concentration of 100 µg/ml in 2 M urea, 0.1 M Tris, 1 mM CaCl (pH 8.3), were mixed with 50 µl/ml of a 0.6 µg/ml solution of

micrococcal nuclease (Sigma) and were incubated at 37°C. After various incubation times, 1 ml aliquots were removed and the reaction was stopped by the addition of 2 ml of cold 10% perchloric acid. The reaction mixtures were then incubated for 10 minutes at 0°C, were centrifuged for 4 minutes at 8000 x G in an Eppindorf 3200 centrifuge, and the absorbance of the supernatant at 260 nm was measured.

b. Digestion of 0.4 M SP-Sephadex fraction with micrococcal nuclease

The 0.4 M SP-Sephadex fraction of S phase HeLa chromosomal proteins, at a concentration of 3.4 μg/ml, was dialyzed for 3 hours each against 2 changes of 100 volumes of 2 M urea, 0.1 M Tris (pH 8.3), 1 mM $CaCl_2$ at 4°C. The dialyzed proteins were incubated for 30 minutes at 37°C with 120 μl/ml of a 0.6 μg/ml solution of micrococcal nuclease in 0.1 M Tris 1 mM $CaCl_2$(pH 8.3) and the reaction was then stopped by the addition of 100 mM EGTA (pH 7.0) to a final concentration of 5 mM. In order to assay the ability of the nuclease treated SP-Sephadex fraction to activate histone gene transcription, 1 mg of G_1 DNA as chromatin was dissociated in 3 M NaCl, 5 M urea, 10 mM Tris, 5 mM EGTA (pH 8.3) and was reconstituted in the presence of 0.3 μg of the treated SP fraction with all solutions containing 5 mM EGTA. The reconstituted chromatin was then transcribed in vitro in the presence of 2 mM EGTA and the purified transcripts were assayed for their ability to hybridize to histone cDNA.

In order to determine if micrococcal nuclease was active in the presence of the 0.4 M SP-Sephadex fraction, in a parallel experiment,

3 µg/ml of ^{3}H-labeled double stranded lambda DNA (obtained from R.P. Boyce, Department of Biochemistry and Molecular Biology, University of Florida) was added to the reaction mixture. After incubation for 30 minutes with micrococcal nuclease, acid precipitable radioactivity was determined by adding 2 volumes of cold 10% TCA and collecting the precipitant on 0.45 micron HA nitrocellulose filters. The filters were then dissolved in 1 ml of cellosolve, and counted in 10 ml of cellosolve-toluene scintillation fluid.

7. Activation of Histone Gene Expression in Heterologous Systems

a. Culture and preparation of chromatin from WI-38 fibroblasts

Human diploid WI-38 fibroblasts grown in monolayer culture in Eagle's basal media (BME) containing 10% fetal calf serum were available in this laboratory. The cells were cultivated in roller bottles at 37°C in an atmosphere of 5% CO_2. The cells are confluent 7 days after plating, but can be stimulated to proliferate by replacing the exhausted growth media with fresh BME containing 20% fetal calf serum. It has previously been shown that under these conditions approximately 60% of the contact-inhibited cells are stimulated to replicate DNA.[219,220] The cells used in this study ranged from passage 28 to 30. Nuclei and chromatin from these cells were prepared as described above for HeLa cells.

b. Preparation of mouse liver chromatin[221]

To obtain mouse liver nuclei and chromatin, 10 adult animals were sacrificed and a total 12.5 grams of liver was removed and minced into 20 volumes of 2.2 M sucrose, 4 mM $MgCl_2$. The minced tissue was then homogenized 10 strokes with a motor driven Potter-Elvehjem teflon homogenizer and filtered through 1 layer of miracloth. The homogenate was centrifuged for 1 hour at 113,000 x G maximum (25,000 rpm) in a Beckman SW 27 rotor at 4°C. The pellet was washed twice in 0.15 M NaCl, 10 mM Tris (pH 8.0) followed each time by centrifugation at 100 x G for 3 minutes. Nuclei were lysed in 60 volumes of double distilled water by gentle homogenization. The chromatin was allowed to swell in an ice bath for 20 minutes and was then pelleted by centrifugation at 12,000 x G for 20 minutes.

c. Preparation of L-929 cell polysomes[221]

Mouse L-929 cells grown in monolayer culture were obtained from Dr. G. E. Gifford of the Department of Immunology and Medical Microbiology, University of Florida. Nonconfluent cultures were harvested with a rubber policeman, washed 3 times in Earle's balanced salt solution, and pelleted by centrifugation at 1000 x G for 3 minutes. The pellet was drained well and resuspended in 10 volumes of 10 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$ (pH 7.4). The cell suspension was transferred to a Dounce homogenizer and after 20 minutes cells were lysed by homogenization with a tight fitting pestle. The homogenate was

centrifuged at 15,000 x G for 15 minutes to pellet nuclei and mitochondria. The supernatant was transferred to 8 ml polycarbonate tubes and centrifuged at 100,000 x G for 90 minutes. All glassware was treated with diethylpyrocarbonate and then autoclaved. The pelleted polyribosomes were resuspended in 1% SDS, 0.1 M NaCl, 10 mM sodium acetate and 0 mM EDTA (pH 5.4). Polysomal RNA was extracted twice with 1 volume of phenol plus 1 volume of chloroform-isoamyl alcohol (24:1) (V/V) followed by 2 extractions with 1 volume of chloroform-isoamyl alcohol. The RNA was precipitated with 2 volumes of ethanol at -20°C, centrifuged at 30,000 x G for 15 minutes and resuspended in 25 mM HEPES, 0.5 M NaCl, 1 M EDTA (pH 7.0) for hybridization analysis.

RESULTS AND DISCUSSION

A. Transcription of G_1 and S Phase Chromatin[13,213]

In HeLa S_3 cells the synthesis of the histone polypeptides is restricted to the S phase of the cell cycle[168-171] and translation[169-171,189] as well as hybridization data[198] indicate that histone mRNAs are associated with polysomes only at this time. One might anticipate that the transcription of histone mRNAs from chromatin is also restricted to the S phase of the cell cycle, but direct evidence for this contention is lacking. To directly assay the availability of histone sequences for transcription in initial studies in this laboratory, we transcribed chromatin *in vitro* and assayed the RNA transcripts for the presence of histone sequences by hybridization with histone cDNA.

1. In vitro Transcription of Chromatin

G_1 and S phase cells were prepared by synchronization of an exponentially growing culture of HeLa S_3 cells as described in Materials and Methods (section B). Chromatin prepared from these cells was then transcribed in a cell-free system using *E. coli* RNA polymerase. After incubation, the reaction mixture was deproteinized by extraction with phenol and chloroform-isoamyl alcohol and the nucleic acids were

precipitated with ethanol. This pellet was then resuspended, and the DNA was degraded by incubation with 40 μg/ml of DNAase I. Following extraction with phenol and chloroform-isoamyl alcohol the aqueous phase containing the RNA transcripts was chromatographed on Sephadex G-50. The transcripts were then precipitated with ethanol and resuspended in 50% formamide, 25 mM HEPES, 0.5 M NaCl, and 1 mM EDTA (pH 7.0). The RNA transcripts were assayed for the presence of histone sequences by annealing at 52°C in 15 μl capillary tubes with 0.04 ng of histone complementary DNA (27,000 dpm/ng). After various incubation times the reaction mixtures were assayed for hybrid formation using single strand specific S_1 nuclease isolated from Aspergillus oryzae. The amount of radioactive DNA resistant to digestion was determined by trichloroacetic acid precipitation.

The kinetics of hybridization of histone complementary DNA and RNA transcripts from G_1 and S phase chromatin are shown in Figure 1. While transcripts from S phase chromatin hybridize with histone cDNA with a $Cr_0t_{\frac{1}{2}}$ of 2×10^{-1} (compared with a $Cr_0t_{\frac{1}{2}}$ of 1.7×10^{-2} for the histone mRNA-cDNA hybridization reaction), there is no hybridization above the background level of 3 to 5% with G_1 transcripts even at a Cr_0t of 100. As can be calculated from the intercept of the double reciprocal plot shown in the inset of Figure 1, the maximal level of hybrid formation between histone cDNA and S phase transcripts is approximately 63%. This is the same as observed in the hybridization reaction between histone cDNA and histone mRNA. Fidelity of the hybrids formed between histone cDNA and transcripts from S phase

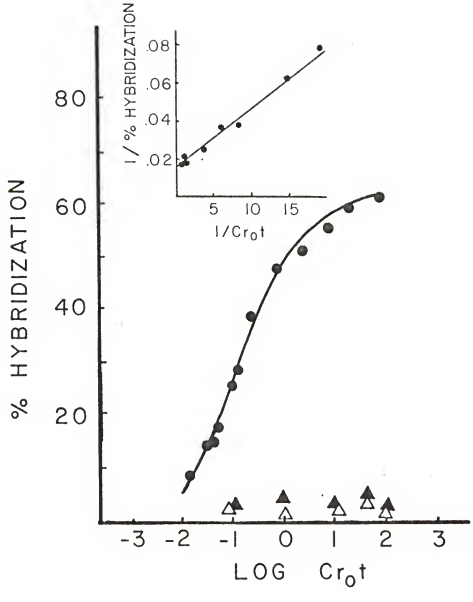

Figure 1. Kinetics of annealing of histone cDNA at 52°C to in vitro transcripts from S phase (●) and G_1 phase (▲) chromatin and to endogenous RNA isolated from S phase chromatin with E. coli RNA as a carrier (△). The inset shows a double reciprocal plot for the annealing of histone cDNA to transcripts from S phase chromatin.

chromatin is identical to the melting temperature of histone mRNA-cDNA hybrids (67°C in 50% formamide, 0.5 M NaCl, 20 mM HEPES (pH 7.0) and 1 mM EDTA). From a comparison of the $Cr_0t_{\frac{1}{2}}$ values of the S phase chromatin transcript--cDNA and histone mRNA-cDNA hybridization reactions, it can be estimated that 8% of the S phase transcripts are histone specific sequences. The figure of 8% is based on the assumption that the histone mRNA preparation consists entirely of histone mRNA sequences. The presence of other sequences in the preparation would require a corresponding reduction in this figure.

2. Assay of Endogenous RNA

RNA synthesized in intact cells may remain associated with chromatin during isolation and, at least in part, account for hybrid formation between in vitro RNA transcripts and complementary DNA for specific genes. This problem has been encountered by several investigators examining regulation of globin gene transcription,[222,223] but has not been seen by all of the workers using this system.[116] It is possible that the extent to which this phenomenon occurs varies with the tissue or cell and the method of chromatin preparation. To determine whether such endogenous RNA sequences account for histone specific sequences which are detected in transcripts from S phase chromatin, the following approach was pursued. S phase chromatin was placed in the in vitro transcription mixture without RNA polymerase and an amount of E. coli RNA equivalent to the amount of RNA transcribed from S phase chromatin was added. RNA was immediately extracted by the same

procedure used for the isolation of *in vitro* RNA transcripts. When this control RNA was annealed with histone cDNA no significant level of hybridization was observed (Figure 1). Additionally, RNA isolated from S phase chromatin in the absence of carrier RNA showed no hybrid formation with histone cDNA. These results indicate that endogenous histone specific sequences associated with S phase chromatin are not contributing significantly to hybridization observed with S phase *in vitro* transcripts. This is consistent with the data of Stein and Stein[224] which indicates that the histone mRNA sequences represent only a small proportion of the total nuclear RNA.

3. Detection of Histone Sequences in G_1 Transcripts

When G_1 chromatin is transcribed in the presence of an amount of histone mRNA equivalent to that transcribed from S phase chromatin and the mixture of G_1 transcripts and added histone mRNA is subsequently isolated, hybridization of histone cDNA occurs at the expected $Cr_o t_{\frac{1}{2}}$ (2×10^{-1}). This result suggests that histone sequences would have been detected in G_1 transcripts if they had been transcribed. The possibility that histone sequences are present in G_1 transcripts but are not detected because they are in a double stranded form due to symmetric transcription is unlikely, since heating the hybridization mixture to 100°C for 10 minutes before incubation has no effect on the hybridization of cDNA to the transcripts.

4. Conclusions

Taken together these results indicate that while the total amount of RNA transcribed from G_1 and S phase chromatin is similar, that histone sequences are efficiently transcribed in vitro from chromatin isolated from S phase cells but that chromatin isolated from G_1 cells does not serve as a template for the transcription of histone sequences.

This cell cycle stage specific transcription of histone sequences is consistent with the tissue specific transcription from chromatin with E. coli RNA polymerase which has been observed for the globin[115,116] and ovalbumin genes.[14,225] It should be stressed, however, that in all of these systems little is known about the accuracy of in vitro initiation, termination or DNA strand selection.

B. Determination of Which Component of Chromatin Is Responsible for Differences in Histone Gene Transcription

1. Reconstitution of Chromatin with Pooled DNA and Histone[13]

Other early studies were directed toward determining which component of chromatin is responsible for the difference in in vitro transcription of histone sequences from G_1 and S phase chromatin. Chromatin was prepared from both G_1 and S phase cells and was then fractionated into DNA, histones and nonhistone chromosomal proteins. To prepare chromosomal proteins, chromatin was first dissociated in 3 M NaCl, 5 M urea, and the DNA was pelleted at 150,000 x G for 36

hours. The proteins were fractionated into histone and nonhistone chromosomal protein fractions by the QAE-Sephadex method of Gilmour and Paul.[109] DNA was prepared from chromatin by the method of Marmur,[215] treated with pancreatic ribonuclease A and pronase and then extracted twice with phenol and chloroform-isoamyl alcohol before use. As shown in Figure 2, the DNA and histones were pooled and chromatin was then reconstituted with the pooled DNA and histone and either G_1 or S phase nonhistone chromosomal proteins. These reconstituted chromatins were transcribed with E. coli RNA polymerase and the purified transcripts were annealed with histone cDNA. While the total amount of RNA transcribed from these reconstituted chromatins was similar, as shown in Figure 3, transcripts from chromatin reconstituted with pooled DNA and histone and S phase nonhistone chromosomal proteins hybridize efficiently with histone cDNA ($Cr_0t_{\frac{1}{2}} = 2 \times 10^{-1}$) whereas transcripts from chromatin reconstituted from pooled DNA and histone and G_1 nonhistone chromosomal proteins do not hybridize significantly with histone cDNA even at a Cr_0t of 100. It should be emphasized that the kinetics and extent of hybridization with the cDNA are the same for transcripts of native S phase chromatin and transcripts from chromatin reconstituted with S phase nonhistone chromosomal proteins.

These results indicate that it is the nonhistone chromosomal protein portion of the chromatin that is responsible for the difference in the transcription of histone sequences in G_1 and S phase chromatin. Such a regulatory role for nonhistone chromosomal proteins is in

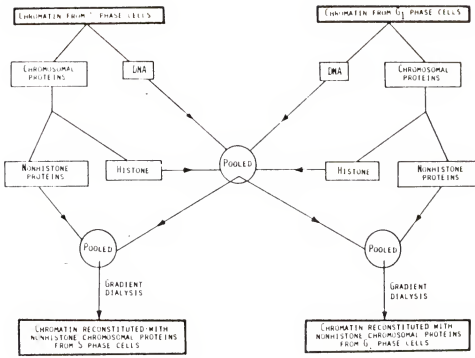

Figure 2. Flow diagram for reconstitution of chromatin with pooled DNA and histone.

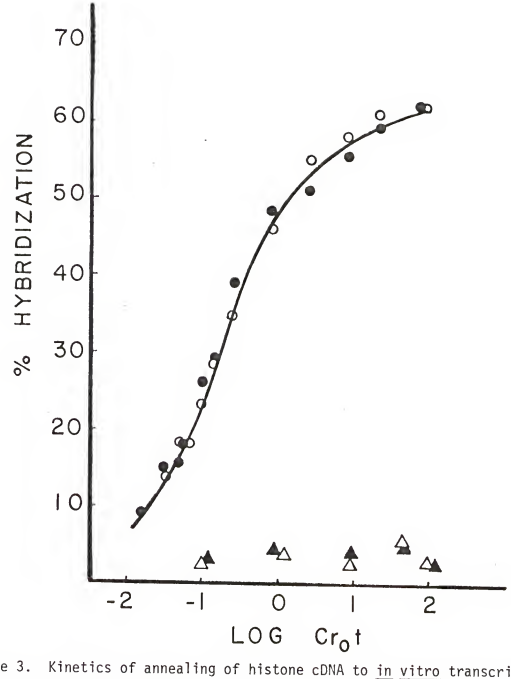

Figure 3. Kinetics of annealing of histone cDNA to in vitro transcripts from chromatin reconstituted with pooled DNA and histone. Histone cDNA was annealed at 52°C with RNA transcripts from chromatin reconstituted with pooled DNA and histone and S phase nonhistone chromosomal protein (○), chromatin reconstituted with pooled DNA and histone and G_1 phase nonhistone chromosomal protein (△), native S phase chromatin (●) and native G_1 phase chromatin (▲).

agreement with results from several laboratories which have indicated that these proteins are responsible for the tissue specific transcription of globin gene[10,11,12,117] and the hormone induced transcription of ovalbumin genes.[14,225]

2. Reconstitution of Chromatin in the Presence of Additional Chromosomal Proteins[226]

In order to examine whether the difference in the in vitro transcription of histone genes from G_1 and S phase chromatin is due to an activator of histone gene transcription present in the S phase chromosomal proteins, a repressor of histone gene transcription present in G_1 phase chromosomal proteins, or both, a series of chromatin reconstitution experiments were performed. If the difference in histone gene activity of G_1 and S phase were due to an activator that is present or operative only in the S phase, one would anticipate that dissociation of G_1 phase chromatin with high salt and urea followed by reconstitution in the presence of increasing amounts of S phase chromosomal protein would result in a progressive increase in the availability of histone genes for transcription. One would not anticipate any major effect on histone gene transcription if S phase chromatin were reconstituted in the presence of G_1 phase chromosomal proteins. In contrast, if the difference in histone gene expression in G_1 and S phase can be accounted for by a repressor of histone gene expression, which is associated with chromatin during the G_1 phase of the cell cycle, one would anticipate that dissociation of S phase

chromatin followed by reconstitution in the presence of increasing amounts of G_1 chromosomal proteins would result in a progressive decrease in the availability of histone genes for transcription. The presence of S phase chromosomal proteins during reconstitution would not be expected to significantly affect the expression of histone genes from G_1 chromatin. If the regulation of histone genes involves both repressors and activators acting in an antagonistic fashion, one would anticipate a more complex, intermediate result.

a. Effect of S phase chromosomal protein on transcription of histone genes from G_1 chromatin

Figure 4 shows the results obtained when chromatin from G_1 cells is dissociated with 5 M urea, 3 M NaCl, 10 mM Tris (pH 8.3), reconstituted in the presence of various amounts of S phase total chromosomal proteins, and the availability of histone genes for transcription assayed. Transcripts from G_1 chromatin reconstituted in the absence of S phase protein show no significant hybrid formation with histone cDNA even at a Cr_0t of 100--the same result as observed with native G_1 chromatin transcripts. In contrast, when G_1 chromatin is reconstituted in the presence of increasing amounts of S phase total chromosomal protein, hybrid formation between transcripts from these reconstituted chromatin preparations and histone cDNA occurs at progressively lower Cr_0t values, suggesting that histone sequences are being made available for transcription. This apparent stimulation of histone gene transcription is not observed when G_1 chromatin is

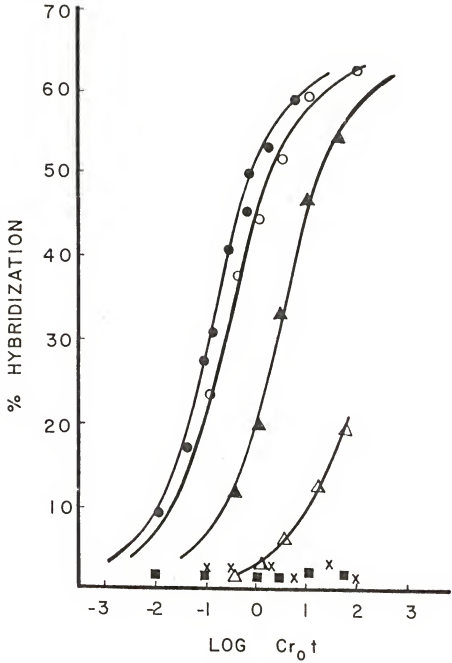

Figure 4. Kinetics of annealing of histone cDNA to <u>in vitro</u> transcripts from G_1 chromatin reconstituted in the presence of various amounts of S phase total chromosomal protein. ^3H cDNA (0.04 ng) was annealed to RNA transcripts from G_1 chromatin reconstituted in the presence of 0 (×), 0.01 (△), 0.10 (▲), or 1.00 (○) mg of S phase total chromosomal protein per mg of G_1 chromatin DNA. cDNA (0.04 ng) was also annealed to RNA transcripts from G_1 chromatin reconstituted in the presence of 1.00 mg of G_1 total chromosomal protein per mg of G_1 chromatin DNA (■) and RNA transcripts from chromatin isolated from S phase cells (●).

dissociated and then reconstituted in the presence of additional G_1 total chromosomal protein--even at a one-to-one ratio of additional G_1 protein to DNA (Figure 4). That the histone genes from G_1 chromatin can be rendered transcribable by S phase total chromosomal proteins to approximately the same degree as observed in chromatin isolated from S phase cells is suggested by the similar kinetics of the hybridization reactions of histone cDNA with transcripts from chromatin isolated from S phase cells ($Cr_0t_{\frac{1}{2}} = 2.0 \times 10^{-1}$) and with transcripts from G_1 chromatin reconstituted with a one-to-one ratio of S phase total chromosomal protein to DNA ($Cr_0t_{\frac{1}{2}} = 3 \times 10^{-1}$). It should be noted that there are no significant differences in the yield of RNA or the recovery of RNA during isolation even though the presence of S phase total chromosomal protein during reconstitution can cause a greater than 1000 fold stimulation in the amount of histone sequences transcribed. To eliminate the possibility that a small amount of nucleic acid present in the S phase chromosomal proteins is responsible for the observed hybridization, either by containing histone sequences or by having the ability to render the histone genes transcribable, the residual nucleic acid was removed from the S phase chromosomal proteins by buoyant density centrifugation in 0.41 mg/ml CsCl, 5 M urea, 10 mM Tris-HCl (pH 8.3) in a SW 50.1 rotor at 35,000 rpm for 48 hours at 4°C. As shown in Figure 5, there is no significant difference in the kinetics of hybridization with histone cDNA of transcripts of either CsCl treated S phase chromosomal proteins or untreated S phase chromosomal proteins.

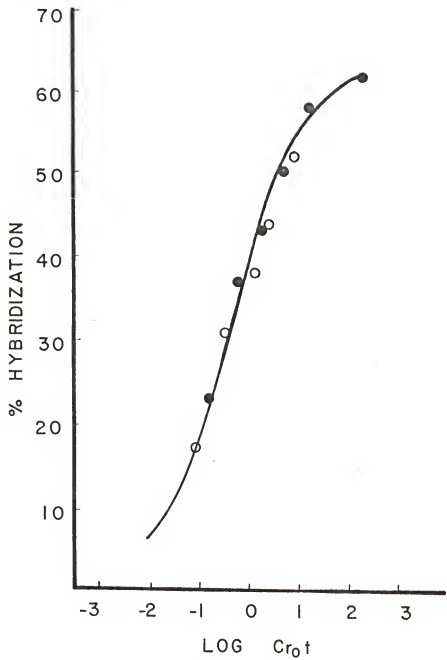

Figure 5. Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 chromatin reconstituted in the presence of S phase total chromosomal protein from which nucleic acid has been removed by centrifugation in CsCl. ^3H cDNA (0.04 ng) was annealed to RNA transcripts from G_1 chromatin reconstituted in the presence of 1.00 mg of CsCl treated S phase total chromosomal protein (○) or 1.000 mg of untreated S phase total chromosomal protein (●) per mg of G_1 chromatin DNA.

In order to determine which component of the S phase chromosomal protein is responsible for the activation of histone gene transcription from G_1 chromatin, S phase chromosomal proteins were fractionated into histones and nonhistone chromosomal proteins with QAE-Sephadex by the method of Gilmour and Paul.[109] As shown in Figure 6, when G_1 chromatin is dissociated and reconstituted in the presence of increasing amounts of S phase nonhistone chromosomal protein, hybrid formation between transcripts from these chromatins and histone cDNA is seen at progressively lower Cr_ot values, indicating a dose-dependent activation of the histone genes of the G_1 chromatin by the S phase nonhistone chromosomal protein. It can be seen that the histone genes from G_1 chromatin can be activated to approximately the same degree as in native S phase chromatin by comparing the kinetics of hybridization of histone cDNA with transcripts from S phase chromatin ($Cr_ot_{1/2} = 2 \times 10^{-1}$) and the kinetics of hybridization of histone cDNA with transcripts from G_1 chromatin reconstituted with a one-to-one ratio of S phase nonhistone protein to DNA ($Cr_ot_{1/2} = 3 \times 10^{-1}$). The fidelity of the hybrids formed between the transcripts and histone cDNA, as well as the validity of comparing $Cr_ot_{1/2}$ values, is suggested by the fact that the T_m of the hybrids in all cases is identical to the T_m of the hybrids formed between histone mRNA and histone cDNA and that the maximal hybridization as estimated by a double-reciprocal plot is equal in all cases to that of the histone mRNA-cDNA hybridization reaction (63%). In contrast, as is also shown in Figure 6, when G_1

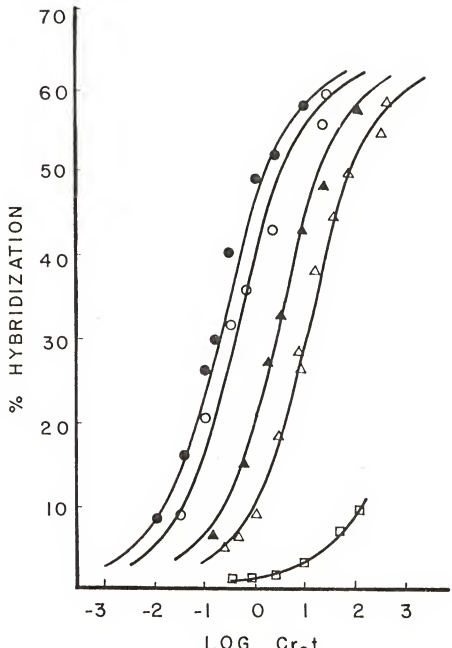

Figure 6. Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 chromatin reconstituted in the presence of either S phase histones or nonhistone chromosomal proteins. ^3H cDNA (0.04 ng) was annealed to RNA transcripts from G_1 chromatin reconstituted in the presence of 0.01 (△), 0.10 (▲), or 1.00 (○) mg of S phase nonhistone chromosomal protein per mg of G_1 DNA as chromatin. cDNA (0.04 ng) was also annealed to RNA transcripts from G_1 chromatin reconstituted in the presence of 1.00 mg of S phase histone per mg of G_1 DNA (□) and transcripts from native S phase chromatin (●).

chromatin is dissociated and then reconstituted in the presence of S phase histones, even at a one-to-one ratio of S phase histone to DNA, only a very small stimulation of the transcription of histone genes is observed. It is unclear whether this low level of histone gene transcription, approximately 0.1% of that seen from G_1 chromatin reconstituted with an equivalent amount of S phase nonhistone chromosomal proteins, is actually due to the S phase histones. It may represent the limits of this procedure for separating the histones and nonhistone chromosomal proteins or it may be due to the presence of approximately 0.1% S phase cells in the G_1 phase cells used for the assay. It should be noted that there were no significant differences among the various chromatin preparations in the yield or recovery of RNA during isolation, even though the presence of S phase nonhistone chromosomal protein during reconstitution could cause a greater than 1000 fold stimulation in the amount of histone sequences transcribed from G_1 chromatin.

b. Effect of G_1 phase chromosomal proteins on transcription of histone genes from S phase chromatin

In order to determine whether G_1 chromatin contains an inhibitor of histone gene transcription that is degraded or inactivated as cells progress from the G_1 to the S phase of the cell cycle, chromatin from S phase cells was dissociated and then reconstituted in the presence of total chromosomal proteins from G_1 phase cells. The ability of transcripts from this reconstituted chromatin preparation to hybridize with histone cDNA was then determined. As shown

in Figure 7, the presence of G_1 total chromosomal protein--even at a one-to-one ratio of G_1 total chromosomal protein to DNA--does not significantly inhibit histone gene transcription from S phase chromatin. This is not to say that there is nothing in G_1 chromosomal protein that can inhibit histone gene transcription, but rather that there is nothing in the G_1 chromosomal protein that can inhibit *in vitro* histone gene transcription in the presence of S phase chromosomal proteins. This would suggest that any additional specific repressor of histone gene expression is lost during isolation, dissociation, fractionation, or reconstitution or that any inhibition of histone gene transcription by G_1 chromosomal proteins can be overridden by S phase chromosomal proteins. As shown in Figure 8, similar results are obtained when S phase chromatin is dissociated and then reconstituted in the presence of G_1 phase nonhistone chromosomal protein. Again the T_m of the hybrids formed and the maximal hybridization are the same as seen in the histone mRNA-cDNA reaction.

These results strongly support the contention that the difference in the *in vitro* transcription of histone genes from G_1 and S phase chromatin is due to the nonhistone chromosomal protein portion of the genome and further show that this difference can be accounted for by a component or components of the S phase nonhistone chromosomal proteins that has the ability to render the histone genes of G_1 phase chromatin available for transcription in a dose-dependent fashion. These results do not indicate which component or components of the S phase nonhistones is responsible for the observed activation or by

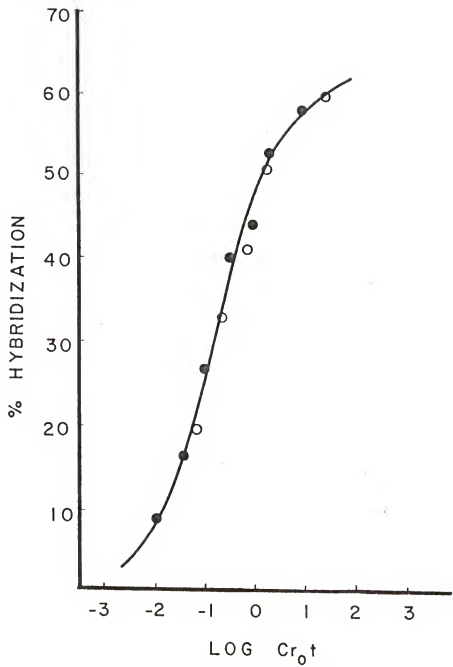

Figure 7. Kinetics of annealing of histone cDNA to in vitro transcripts from S phase chromatin reconstituted in the presence of G_1 phase total chromosomal proteins. ^3H cDNA (0.04 ng) was annealed to RNA transcripts from S phase chromatin reconstituted in the presence of 1.00 mg of G_1 phase total chromosomal protein per mg of S phase DNA (○). cDNA was also annealed to transcripts from native S phase chromatin (●).

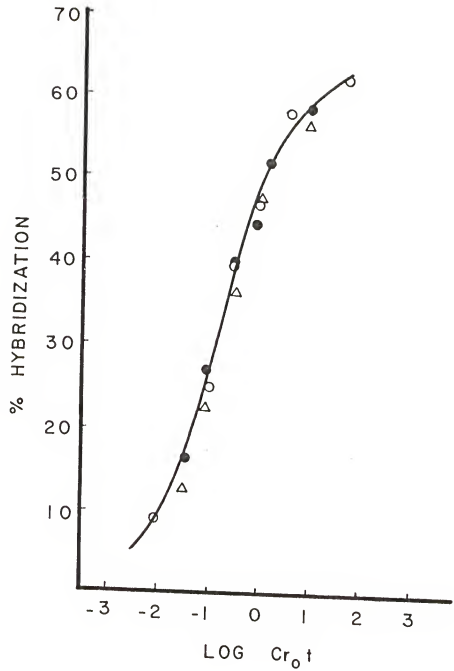

Figure 8. Kinetics of annealing of histone cDNA to in vitro transcripts from S phase chromatin reconstituted in the presence of G_1 phase non-histone chromosomal proteins. ^3H cDNA (0.04 ng) was annealed to RNA transcripts from S phase chromatin reconstituted in the presence of 0.10 (△) or 1.00 (○) mg of G_1 phase nonhistone chromosomal proteins per mg of S phase DNA. cDNA was also annealed to transcripts from native S phase chromatin (●).

what mechanism the activation is achieved, but they do provide an assay by which this histone gene activator can be purified and characterized.

C. Purification of Histone Gene Activator[227]

In order to determine which component(s) of the S phase nonhistone chromosomal proteins is responsible for the ability of these proteins to activate histone gene transcription from G_1 phase chromatin, the S phase chromosomal proteins were fractionated using ion exchange and gel filtration chromatography.

1. Fractionation on QAE-Sephadex

QAE-Sephadex is commonly used in batch procedures to separate the histones and nonhistone chromosomal proteins,[109] but as shown in Figure 9, it can also be used to fractionate the nonhistone chromosomal proteins. Total S phase chromosomal protein, from which nucleic acids have been removed by ultracentrifugation, were dialyzed against 5 M urea, 10 mM Tris (pH 8.3) and were loaded on a column of QAE-Sephadex A-25, previously equilibrated with the same buffer. The proteins were then eluted with 2 column volumes each of 5 M urea, 10 mM Tris (pH 8.3) containing 0.1, 0.25, 0.5 and 3.0 M NaCl. As shown in Figure 10, the histones and approximately 10% of the nonhistone chromosomal proteins are not bound and are eluted in the void volume, whereas a complex but electrophoretically distinct class of nonhistone chromosomal

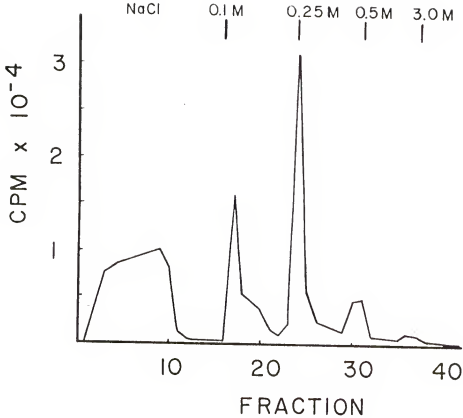

Figure 9. Elution profile of ^{3}H-leucine labeled S phase total chromosomal protein from QAE-Sephadex. Proteins were loaded in 5 M urea, 10 mM Tris (pH 8.3) and were eluted with this buffer containing 0.10 M, 0.25 M, 0.50 M and 3.0 M NaCl.

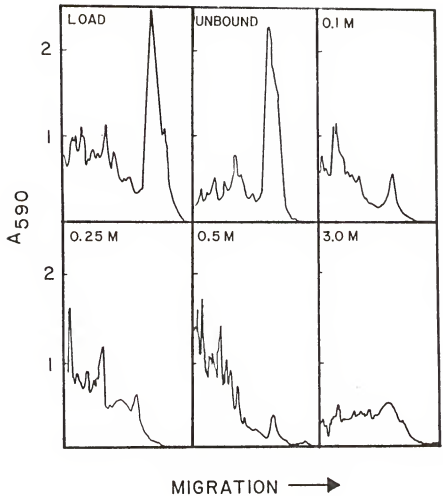

Figure 10. SDS polyacrylamide gel profile of S phase chromosomal proteins fractionated with QAE-Sephadex.

proteins is eluted by each salt concentration. The total recovery of protein from the column is 50-75%.

In order to determine the ability of each of the QAE fractions to render histone genes available for transcription, 3 mg of chromatin from G_1 phase cells (containing approximately 1 mg DNA) was dissociated with 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) and was reconstituted in the presence of 100 μg of each of the QAE fractions. The reconstituted chromatin was when transcribed in vitro with E. coli RNA polymerase, and the isolated transcripts were assayed for histone mRNA sequences by hybridization to histone cDNA. As shown in Figure 11, transcripts from G_1 chromatin reconstituted in the presence of the unbound fraction or the material eluted with 0.1, 0.25, or 3 M NaCl from QAE-Sephadex did not show significant hybrid formation with histone cDNA even at a Cr_0t of 100--the same results observed with transcripts from native G_1 chromatin. In contrast, even though the total amount of RNA transcribed was similar, transcripts from G_1 chromatin reconstituted in the presence of 0.5 M fraction hybridized efficiently with histone cDNA ($Cr_0t_{\frac{1}{2}} = 4 \times 10^{-1}$). The 0.5 M fraction contains approximately 7% of the protein eluted from the QAE column. Since the total recovery of protein ranges from 50-75% this corresponds to 3-5% of the total S phase chromosomal proteins.

2. Fractionation on SP-Sephadex

In order to further purify the histone gene activator, the 0.5 M QAE fraction was chromatographed on SP-Sephadex as shown in

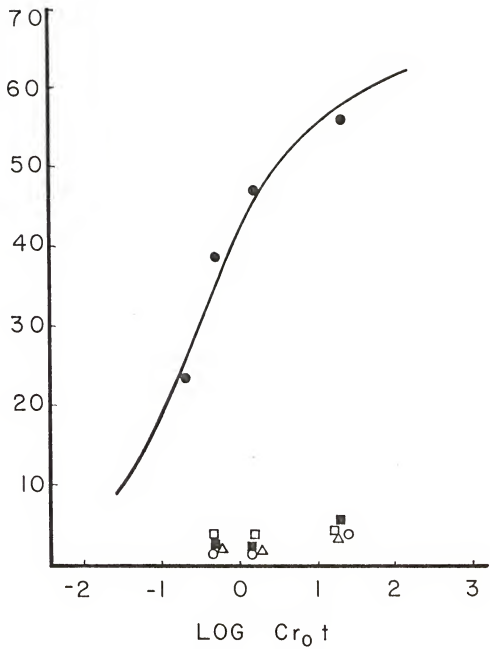

Figure 11. Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 chromatin reconstituted in the presence of S phase HeLa chromosomal proteins fractionated on QAE-Sephadex. ^3H cDNA was annealed at 52°C to transcripts from 1 mg G_1 DNA as chromatin reconstituted in the presence of 100 µg of S phase HeLa chromosomal proteins eluted from QAE-Sephadex by 5 M urea, 10 mM Tris (pH 8.3) containing 0 M (□), 0.10 M (■), 0.25 M (●), 0.50 M (△) and 3.0 M NaCl.

Figure 12. The proteins were titrated to pH 5.2 with 1 M sodium acetate (pH 4.5), were dialyzed against 5 M urea, 0.1 M NaCl, 0.2 M sodium acetate (pH 5.2) and were loaded on a column of SP-Sephadex C-25, previously equilibrated with the same buffer. The proteins were then eluted with 2 column volumes each of 5 M urea, 0.2 M sodium acetate (pH 5.2) containing 0.2 M and 0.4 M NaCl and then with two column volumes of 5 M urea, 3 M NaCl, 100 mM Tris-HCl (pH 8.3). The total recovery of these proteins is approximately 50%. The electrophoretic profiles of these proteins on a SDS polyacrylamide slab gel are shown in Figure 13.

In order to assay the ability of the SP fractions to render histone genes available for transcription, 1 mg of G_1 DNA as chromatin was dissociated with 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) and was reconstituted in the presence of 10 µg of each of the SP fractions. The reconstituted chromatin was then transcribed *in vitro* and the isolated transcripts were assayed for histone mRNA sequences by hybridization to histone cDNA. Although the total amount of RNA transcribed from these reconstituted chromatins is similar, as shown in Figure 14, only the fraction reconstituted in the presence of 10 µg of 0.4 M NaCl SP fraction hybridizes with histone cDNA ($Cr_0t_{1/2}$ = 2×10^{-1}). The 0.4 M SP fraction contains only approximately 10% of the protein loaded on the column.

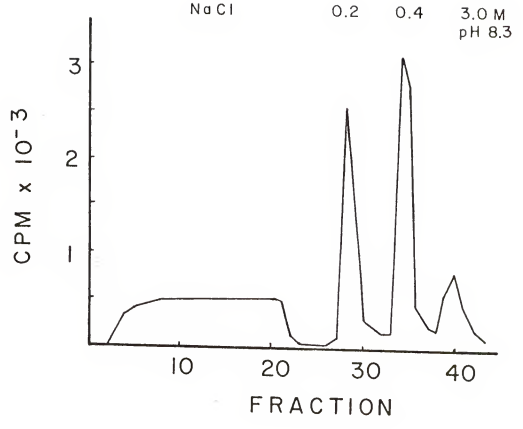

Figure 12. Elution profile of ^{3}H-leucine labeled protein eluted from QAE-Sephadex by 0.4 M NaCl when chromatographed on SP-Sephadex. Proteins were loaded in 5 M urea, 0.2 M sodium acetate, 0.1 M NaCl (pH 5.2) and were eluted with this same buffer containing 0.2 and 0.4 M NaCl and then with 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3).

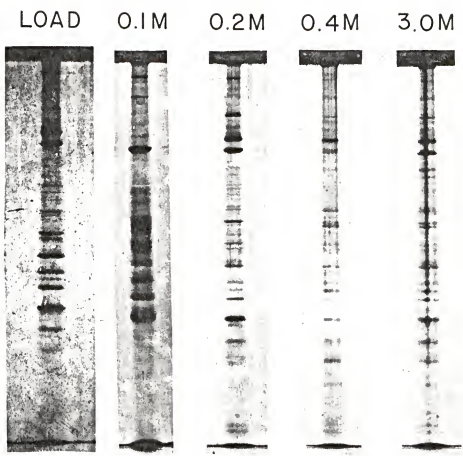

Figure 13. SDS polyacrylamide gel profile of the material eluting from QAE-Sephadex with 0.5 M NaCl when further fractionated with SP-Sephadex.

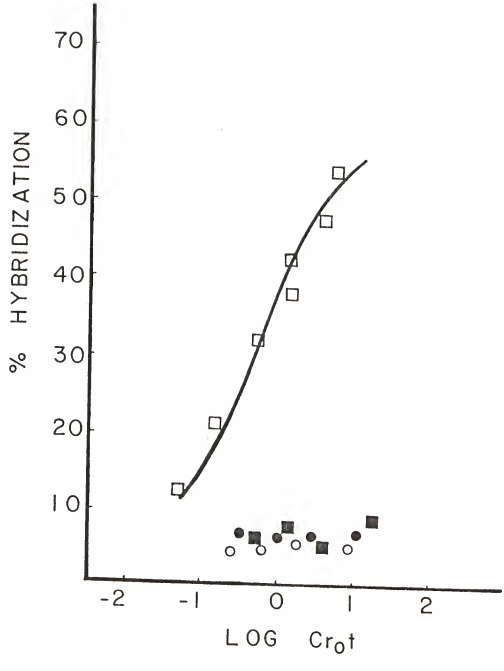

Figure 14. Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 HeLa chromatin reconstituted in the presence of fractions from SP-Sephadex. ^3H cDNA was annealed at 52°C to transcripts from 1 mg of DNA as chromatin reconstituted in the presence of 10 µg of protein with 0.1 M (○), 0.2 M (●), 0.4 M (□) and 3.0 M (■) NaCl.

3. Chromatography on G-100

As shown in Figure 13, the 0.4 M SP fraction is very heterogenous, covering the entire molecular weight range of an 8.75% acrylamide SDS slab gel (16,000-200,000 molecular weight). In order to take advantage of this wide molecular weight range, the 0.4 M SP fraction was chromatographed on Sephadex G-100. The proteins were titrated to pH 8.3 with 1.0 M Tris-base and were made 3 M NaCl by addition of solid NaCl. Figure 15 shows the profile obtained when 3.4 μg of the SP fraction is chromatographed on a 1.5 by 27 cm column of Sephadex G-100 equilibrated with 5 M urea, 3 M NaCl, 10 mM Tris (pH 8.3). The total recovery of protein was greater than 90%.

In order to determine where the activity eluted from the column, fractions 8 and 9, 10 and 11, 12 and 13, and 14 through 16 were pooled and each pool was assayed for its ability to activate histone gene transcription. One milligram of G_1 DNA as chromatin was reconstituted with 1 ml of each of the pooled fractions. The reconstituted chromatin was then transcribed *in vitro* and the isolated transcripts were assayed by hybridization to histone cDNA. As shown in Figure 16 only transcripts from chromatin reconstituted in the presence of the pool containing fractions 10 and 11 hybridized with histone cDNA ($Cr_0t_{\frac{1}{2}} = 5 \times 10^{-1}$). Fractions 10 and 11 contain approximately 12% of the protein applied to the column. The 1 ml of this pooled fraction which was assayed for its ability to activate histone gene transcription contained approximately 0.1 μg of protein.

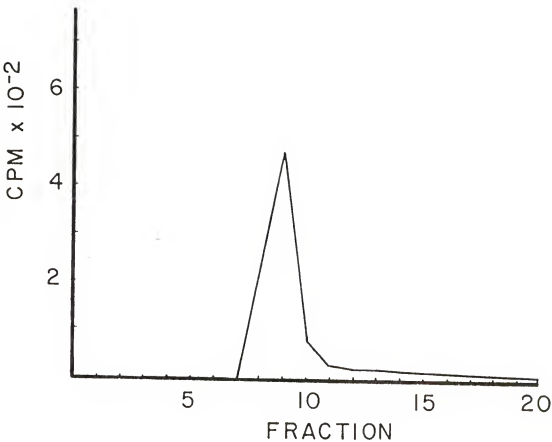

Figure 15. Elution profile of ^{3}H-leucine labeled protein eluted from SP-Sephadex by 0.4 M NaCl when chromatographed on Sephadex G-100. The 0.4 M SP-Sephadex fraction (3.4 μg) was chromatographed in 3 M NaCl, 10 mM Tris (pH 8.3) on a 1.5 x 27 cm column of Sephadex G-100 at 4^{o} and 2 ml fractions were collected.

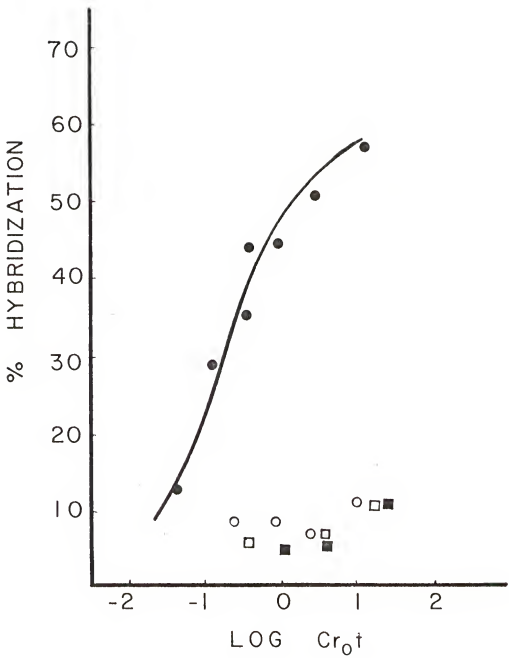

Figure 16. Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 chromatin reconstituted in the presence of fractions from Sephadex G-100. ^{3}H cDNA was annealed at 52^{o}C to transcripts from 1 mg of G_1 DNA as chromatin reconstituted in the presence of 1.0 ml of fractions 8 and 9 (○), 10 and 11 (●), 12 and 13 (□) and 14-16 (■).

4. Assessment of Protein Purification

In order to assess the degree of purification it is necessary not only to be able to determine whether a given fraction has activity but also to determine how much activity it has. To do this we can take advantage of the fact that S phase chromosomal proteins can activate histone gene transcription from G_1 phase chromatin in a dose dependent fashion. The dose response curve obtained when transcripts from G_1 chromatin reconstituted in the presence of various amounts of S phase total chromosomal proteins are hybridized with histone cDNA is shown in Figure 17. Using this as a standard, the relative activity of the purified fractions can be calculated by comparing the amount of a purified fraction required to activate histone gene transcription to a given extent, with the amount of S phase total chromosomal protein required to achieve this same degree of activation. While this approach provides a method of quantitating the amount of histone gene activator activity, and is the first such assay developed to quantitate the ability of chromosomal protein fractions to activate the transcription of specific genes, it should be emphasized that this is not a precise method. Analysis of the ability of chromosomal protein fractions to activate histone gene transcription from G_1 phase chromatin requires a large number of steps, requires the quantitation of small amounts of material, and ultimately involves the measurement of the kinetics of hybridization of transcripts with histone cDNA. The rate of such RNA-DNA hybridization is analyzed using a logarithmic scale and is

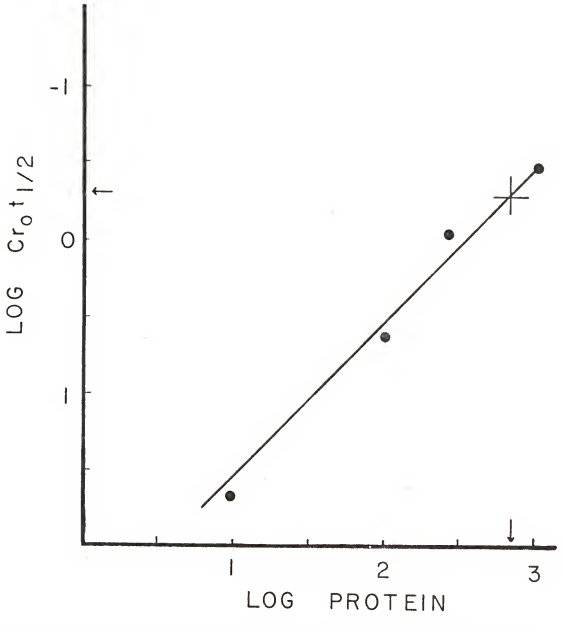

Figure 17. Dose response curve for transcription of histone sequences from G_1 chromatin reconstituted in the presence of S phase total chromosomal protein. G_1 chromatin was dissociated and then reconstituted in the presence of various amounts of S phase total chromosomal protein. Transcripts from these reconstituted chromatins were then annealed at 52°C with histone cDNA. The logarithm of the $Cr_o t_{½}$ of this hybridization reaction is plotted versus the logarithm of the amount of S phase total chromosomal protein added per mg G_1 DNA as chromatin. The small arrows show that one would anticipate that transcripts from 1 mg of G_1 chromatin reconstituted in the presence of 630 μg (antilog of 2.8) of S phase total chromosomal proteins would hybridize with histone cDNA with a $Cr_o t_{½}$ of-0.3.

strongly influenced by a number of factors including the presence of even small amounts of protein or heavy metal ions.

The G-100 fraction contains only approximately 0.04% of the total S phase chromosomal proteins. If this fraction contains all of the histone gene activator activity present in total S phase chromosomal proteins one would anticipate that a given amount of the fraction would have a 2500 fold (1/0.0004) greater ability to activate histone gene transcription than the same amount of total S phase chromosomal protein.

When the G-100 fraction is assayed for its ability to activate histone gene transcription, it is found that transcripts from 1 mg of G_1 DNA as chromatin reconstituted in the presence of 0.1 µg of the G-100 fraction hybridize with histone cDNA with a $Cr_0t_{\frac{1}{2}}$ of 5×10^{-1} ($\log Cr_0t_{\frac{1}{2}} = -0.3$). As can be seen in Figure 17 one would anticipate that it would require 630 µg (antilog of 2.8) of total S phase chromosomal proteins to activate histone gene transcription to the same extent as 630 µg of total S phase chromosomal proteins. Even though the G-100 fraction contains only 0.04% as much protein, one can account for all of the activity present in the total S phase chromosomal proteins by that present in this fraction. These results would, in fact, indicate that there is approximately 2.5 times more activity in the G-100 fraction than in the total S phase chromosomal proteins.

Similar calculations can be performed at each stage of the purification. In such calculations, however, it is important to

realize that while the assay is approximately linear over the range shown in Figure 17, it can be saturated. As shown in Figure 18, transcripts from 1 mg of G_1 DNA as chromatin reconstituted in the presence of 0.1 μg of the SP fraction hybridize with histone cDNA with $Cr_0t_{\frac{1}{2}}$ of approximately 2.5, and transcripts from chromatin reconstituted in the presence of 1.0 μg of the SP fraction hybridize with a $Cr_0t_{\frac{1}{2}}$ of approximately 2×10^{-1}. When the G_1 chromatin is reconstituted in the presence of 10 μg of the SP fraction, however, the transcripts still hybridize with a $Cr_0t_{\frac{1}{2}}$ of approximately 2×10^{-1}. These results indicate that even if G_1 chromatin is reconstituted in the presence of a large excess of histone gene activator that its transcripts will not hybridize with histone cDNA with a $Cr_0t_{\frac{1}{2}}$ of less than approximately 2×10^{-1}. This is the same $Cr_0t_{\frac{1}{2}}$ that is seen when transcripts from native S phase chromatin are hybridized with histone cDNA.

Table 2 shows the amount of protein and relative activity of this protein at each step of the fractionation of S phase total chromosomal proteins. If one examines total activity (that obtained by multiplying the percent initial protein by the relative activity) it can be seen that there is at least as much, and may actually be more, histone gene activator activity present in the purified fractions than in the total S phase chromosomal proteins. It should be noted that the total recovery of protein from the QAE and SP-Sephadex columns was low (50-75%) and it is not known whether additional histone gene activator activity was present in the proteins that were lost. It may be significant in this regard, however, that Thomson et al.[228] have

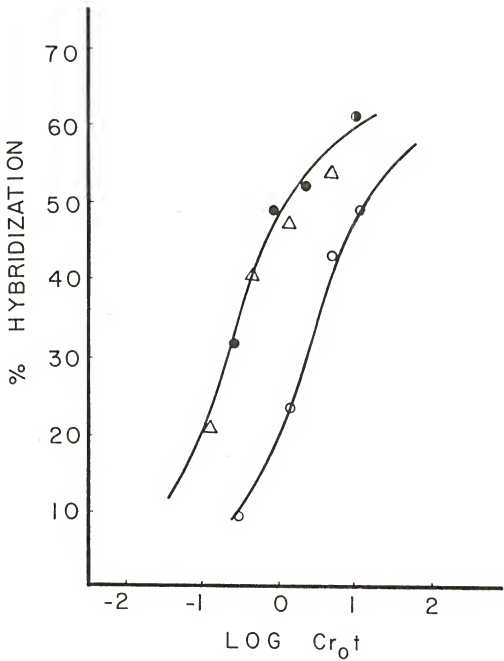

Figure 18. Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 chromatin reconstituted in the presence of various amounts of the 0.4 M SP-Sephadex fraction. ^3H cDNA was annealed at 52°C to transcripts from G_1 chromatin reconstituted with 0.1 (○), 1.0 (△), and 10 (●) µg of the 0.4 M SP-Sephadex fraction per mg G_1 DNA as chromatin.

Table 2

Relative activity of S phase chromosomal protein fractions

Fraction	Protein (mg)	% Initial protein	Relative activity	Total activity
total	20	100	1	100
0.5 M QAE	0.60	3	52	156
0.4 M SP	0.066	0.33	1260	415
G-100	0.008	0.04	6300	252

^{3}H-leucine labeled total chromosomal proteins were fractionated as described in Materials and Methods. The ability of the fractions to activate histone gene transcription was assayed by reconstituting the fractions with G_1 chromatin. When 1 mg of G_1 chromatin was reconstituted in the presence of 6 µg of the 0.5 M QAE fraction, in vitro transcripts from the reconstituted chromatin hybridized with histone cDNA with a $Cr_0t_{\frac{1}{2}}$ of 1. When 0.1 µg of the 0.4 M SP and G-100 fractions was assayed by this procedure the hybridization reactions proceeded with a $Cr_0t_{\frac{1}{2}}$ of 0.5 and 2.5, respectively. Relative activities of the fractions were calculated by comparing these results with the dose response curve for activation of histone gene transcription by total S phase chromosomal protein (shown in Figure 17). Total activities were calculated by multiplying the relative activity of the fractions by the percent of the initial protein they contained. It should be noted that approximately the same amount (80-100 µg/mg G_1 DNA as chromatin) of RNA was recovered as purified transcripts for all samples.

shown that when S phase chromatin is dissociated in 1 M NaCl and is then diluted with 2 volumes of water, that while the DNA, histones, and part of the nonhistone chromosomal proteins precipitate, that the histone gene activator activity remains soluble.

The apparent increase in the amount of histone gene activator may be due to several factors. One source of this apparent increase in activity may be the large error inherent in this type of assay. In addition, the quantitation of proteins during fractionation is based on labeling with ^{3}H-leucine. The cells were labeled for 2 generations before synchronization with 0.2 μCi/ml ^{3}H-leucine and the same amount of leucine was present in all the media used for synchronization. It is not known, however, whether the specific activity of all fractions is exactly the same, particularly since only one amino acid was used for labeling. It should also be noted that it is not known whether the shape of the dose response curve is exactly the same for the purified fraction as for total chromosomal proteins.

One factor which is not thought to be involved is differences in the histone sequences transcribed. There are no reproducible differences in the maximal percent hybridization and, as shown in Figure 19, the hybrids formed between histone cDNA and transcripts from G_1 chromatin reconstituted with the SP or G-100 fractions show the same melting temperature as transcripts from G_1 chromatin reconstituted in the presence of S phase total chromosomal proteins.

If there is an increase in the amount of histone gene activator activity present in purified fractions this may be due to the removal

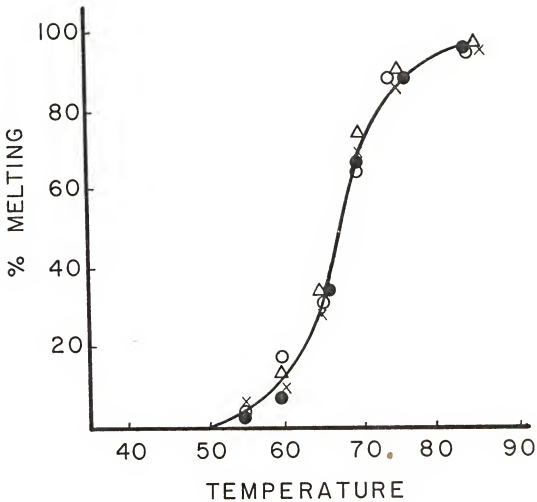

Figure 19. Melting profile of hybrids formed between histone cDNA and histone mRNA (X) and between histone cDNA and transcripts from 1 mg of G_1 DNA as chromatin reconstituted in the presence of 1000 µg of total S phase chromosomal proteins (O), 10 µg of the 0.4 M SP-Sephadex fraction (●) and 0.1 µg of the G-100 fraction(△).

of something which inhibits histone gene transcription or which inhibits the ability of the S phase nonhistone chromosomal proteins to activate histone gene transcription. Such inhibition could be due to a specific inhibitor of histone genes or might be nonspecific. When chromatin is reconstituted with a given amount of histone gene activator using total S phase chromosomal proteins or the purified fractions, the conditions are not exactly comparable. When one uses total S phase chromosomal proteins, much more protein must be added and this protein contains many species not present in the purified fraction. This "additional" protein may nonspecifically inhibit the activation or transcription of the histone genes.

Histone gene activator activity can also be purified from unsynchronized log phase HeLa cells. When total log phase chromosomal proteins are fractionated by the procedure used for the S phase chromosomal proteins, the G-100 fraction is found to contain 0.06% of the protein. When this fraction is assayed for its ability to activate histone gene transcription it is found that when 1.0 mg of G_1 DNA as chromatin is reconstituted in the presence of 0.1 μg of the log phase G-100 fraction that transcripts from the reconstituted chromatin hybridized with the histone cDNA with a $Cr_0t_{\frac{1}{2}}$ of approximately 3 (log $Cr_0t_{\frac{1}{2}}$ = 0.5). Using the dose response curve for total S phase chromosomal proteins shown in Figure 17, one can calculate that the log phase G-100 fraction has a relative activity of approximately 1000 since one would anticipate that it would require 100 μg of total S phase chromosomal proteins to activate histone gene transcription to

the same degree as 0.1 μg of the log phase fraction. In interpreting this relative activity, however, it is very important to realize that the relative activity of log phase total chromosomal proteins is only approximately half that of total S phase chromosomal proteins. This is consistent with the fact that only approximately half the cells in an unsynchronized log phase culture are in S phase and that transcripts from log phase chromatin contain only approximately half as much histone mRNA as transcripts from native S phase chromatin. Since the log phase G-100 fraction contains approximately 0.06% of total log phase chromosomal proteins, but this purified protein is increased in relative activity by approximately 2000 fold, one can calculate that there is approximately 1.2 times as much histone gene activator activity in the purified fraction as was present in the unfractionated log phase chromosomal proteins.

The log phase G-100 fraction contains a larger percentage of the total chromosomal proteins than does the S phase G-100 fraction (0.06 and 0.04%, respectively). Even taking this into account, both the increase in relative activity and the total recovery of activity during isolation are a factor of 2 greater for the S phase G-100 fraction than for the log phase G-100 fraction. This difference may simply be due to the inaccuracy of the assay system, but it cannot be ruled out that it is due, at least in part, to specific factors from other phases of the cell cycle in the log phase protein.

Taken together these results indicate that a component(s) of the nonhistone chromosomal proteins which has the ability to activate

histone gene transcription from G_1 phase chromatin can be isolated from either S phase or unsynchronized log phase cells. This partially purified fraction contains only 0.04-0.06% of the total chromosomal proteins but has sufficient ability to activate histone gene transcription to account for all of the activity present in the total chromosomal proteins. Precise quantitation of the amount of activity in these fractions is difficult, however, since the error involved in these assays is large and several components of the system are poorly understood at this time.

D. Characterization of Histone Gene Activator[221,227]

1. Electrophoresis

Electrophoretic analysis of the partially purified chromosomal protein fractions presents a number of problems since there is only a very small amount of material, the proteins are very dilute, and since the buffers used in the fractionation contain 5 M urea and large amounts of salt. In order to deal with these problems a modification of a procedure of Thomas and Kornberg[229] has been used. In this modified procedure the proteins are dialyzed against 1 M urea, 0.5 M NaCl and are then precipitated with 20% trichloroacetic acid. The precipitate is then recovered by centrifugation, is washed two times with acetone, and is then dissolved in 30 µl of the electrophoresis buffer by boiling for 2 minutes. Using this procedure it has been possible to obtain reproducible SDS gel profiles for proteins present in concentrations as low as 20 µg/ml. Even using this procedure, however,

it has unfortunately not been possible to obtain an electrophoretic profile of the active fraction from the G-100 column. The SDS polyacrylamide slab gel profiles of total chromosomal protein and the active fraction from the QAE-Sephadex and SP-Sephadex columns are shown in Figure 20. Although the QAE and SP fractions contain only 3 and 0.3% respectively of the total chromosomal protein, they still have very complex electrophoretic profiles which cover the entire molecular weight range of an 8.75% acrylamide SDS slab gel (16,000-200,000 daltons). Not only is the electrophoretic profile of the 0.4 M SP fraction very heterogeneous, but also if one compares this profile to that of the material eluted from the SP column by 0.2 M NaCl or 3 M NaCl at pH 8.3, as shown in Figure 13, one can see that while the electrophoretic profiles are slightly different, essentially all of the bands present in the 0.4 M SP fraction are also found in one or both of the other fractions (neither of which has the ability to activate histone gene transcription from G_1 chromatin). These results would seem to indicate that while the histone gene activator has been substantially purified it may still represent only a minor component of even the most purified fraction.

2. Molecular Weight

In order to determine the approximate molecular weight of histone gene activator, advantage can be taken of the fact that chromatography on a gel filtration resin such as Sephadex G-100 is a function of molecular size. When purified proteins of known

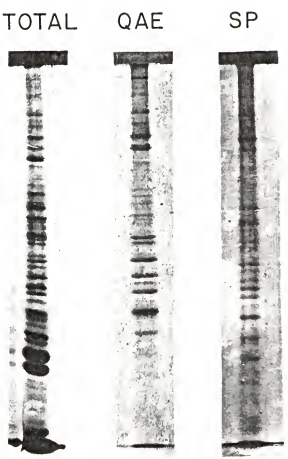

Figure 20. SDS polyacrylamide slab gel electrophoretic profile of S phase chromosomal protein fractions. Total S phase chromosomal proteins, the material eluted from QAE-Sephadex with 0.5 M NaCl, and the material eluted from SP-Sephadex with 0.4 M NaCl were electrophoresed on 8.75% polyacrylamide slab gels and were stained with Coomassie Brilliant Blue.

molecular weight are chromatographed on a 1.5 x 28 cm column of G-100 in 5 M urea, 3 M NaCl, 10 mM Tris (pH 8.3) the results shown in Figure 21 are obtained. These data can be used to calibrate the column since as shown in Figure 22 there is an approximately linear relationship between the log of the molecular weight of these proteins and their elution constant (K_{av}). The elution constant is defined by the equation:

$$K_{av} = \frac{V_E - V_0}{V_T - V_0}$$

where V_E is the elution volume of the protein, V_0 is the void volume and V_T is the total volume of the resin bed.

When total S phase chromosomal proteins are chromatographed on Sephadex G-100 in 5 M urea, 3 M NaCl, 10 mM Tris (pH 8.3), the results in Figure 23 are obtained. In order to determine which of the Sephadex G-100 fractions have the ability to activate histone gene transcription, fractions 8 and 9, 10 and 11, 12 and 13, and 14 through 17 were pooled and 10 ml of each pool was assayed for its ability to activate histone gene transcription from 1 mg G_1 DNA as chromatin. When the transcripts from these reconstituted chromatins are hybridized with histone cDNA as shown in Figure 24 only transcripts from chromatin reconstituted in the presence of fractions 12 and 13 hybridize with histone cDNA. These fractions contain proteins with elution constants between 0.16 and 0.27. This corresponds to a molecular weight range of approximately 40,000-60,000 daltons.

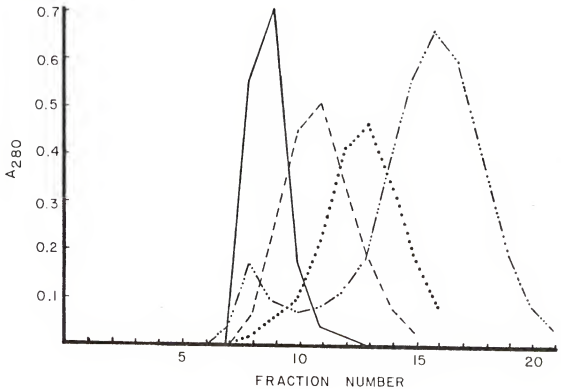

Figure 21. Chromotography of blue dextran and standard proteins on Sephadex G-100. Two ml of 2 mg solutions of Blue Dextran 200 (——), Bovine Serum Albumin (----), ovalbumin (·····) and whale skeletal muscle myoglobin (-...-...-...) were chromatographed in 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) on a 1.5 x 27 cm column of Sephadex G-100 and 2 ml fractions were collected.

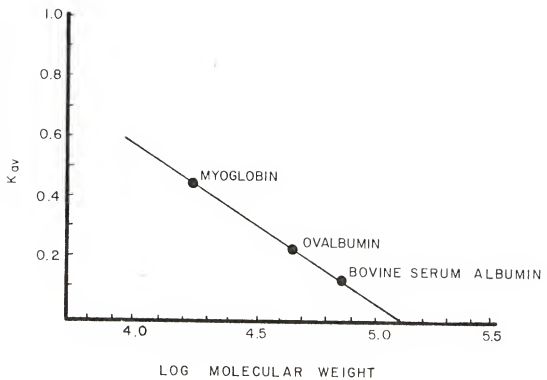

Figure 22. Calibration curve for Sephadex G-100. The elution constant (K_{av}) is defined as:

$$K_{av} = \frac{V_E - V_0}{V_T - V_0}$$

When V_E is the elution volume, V_0 is the void volume and V_T is the total volume of the resin bed. The elution profiles of these proteins are shown in figure 21.

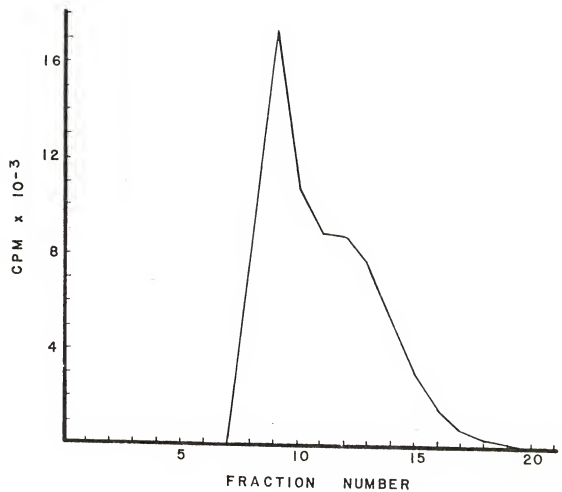

Figure 23. Chromatography of total S phase chromosomal proteins on Sephadex G-100. S phase total chromosomal proteins (2 ml of 2.5 mg/ml solution) were chromatographed on a 1.5 x 27 cm column of Sephadex G-100 in 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) and 2 ml fractions were collected.

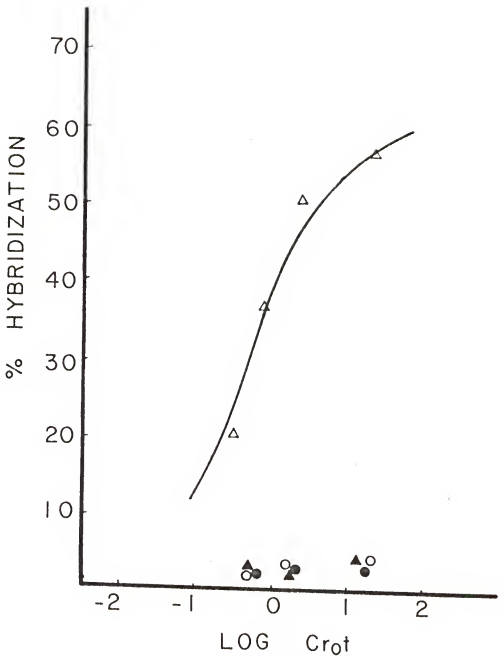

Figure 24. Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 chromatin reconstituted in the presence of S phase total chromosomal proteins which have been fractionated on Sephadex G-100. ^3H cDNA was annealed at 52°C to transcripts from 1 mg of G_1 DNA as chromatin reconstituted in the presence of 100 µg of fractions 8 and 9 (○), 10 and 11 (●), 12 and 13 (△) and 14-16 (▲).

Comparable results are obtained when 0.4 M SP fraction is chromatographed on Sephadex G-100. As was shown in Figures 15 and 16 when these proteins were chromatographed in Sephadex G-100 only the material in fractions 10 and 11 have the ability to activate histone gene transcription. For this column, fractions 10 and 11 contain material with an elution constant between 0.11 and 0.23. This corresponds to a molecular weight range of approximately 45,000-80,000 daltons.

It should be stressed that these are only apparent molecular weights on Sephadex G-100. If the proteins are not approximately spherical or if they interact with each other or with the resin the actual molecular weight may be very different. For example, when a mixture of the five histones is chromatographed on Sephadex G-100 under these same conditions, they have an apparent molecular weight of approximately 55,000.

3. Binding of Chromosomal Fraction to Chromatin During Reconstitution

These studies have demonstrated that when G_1 chromatin is reconstituted in the presence of S phase chromosomal protein fractions that histone genes are made available for transcription. These studies, however, do not indicate how much of the added chromosomal protein fraction is incorporated into the reconstituted chromatin. In order to approach this problem, 8 µg of ^{3}H-leucine labeled 0.4 M SP fraction was added to 1 mg G_1 DNA as chromatin. One-half of this chromatin was immediately made 1% SDS, boiled for 5 minutes, and frozen. The other

half was reconstituted by the usual procedure and the reconstituted chromatin and the supernatent from the reconstitution were recovered and were treated with SDS. When aliquots of these samples were counted, it was found that the reconstituted chromatin contained only 76% of the labeled protein, but also contained 76% of the DNA. The remainder of the label and the DNA were in the supernatent fraction. The unreconstituted chromatin, reconstituted chromatin, and supernatent were found to contain 8,220, 7,660 and 7,800 cpm/mg DNA respectively, indicating that the SP fraction had apparently been incorporated into the reconstituted chromatin.

The reconstituted and unreconstituted chromatin samples were then analyzed by SDS slab gel electrophoresis. As shown in Figure 25, the electrophoretic profiles of these two chromatin preparations are very similar, indicating that significant degradation during reconstitution did not occur. When these gels are sliced and counted, the profile of the ^{3}H labeled SP fraction in reconstituted and unreconstituted chromatin is similar. This would indicate that there are no major differences between the molecular weight profile of the SP fraction that was incorporated into chromatin during reconstitution and that of the total SP fraction, but it should be noted that small differences would not be detected by this procedure.

4. Density in Cesium Chloride

One of the most fundamental questions that might be asked about the molecules which regulate the transcription of the histone gene

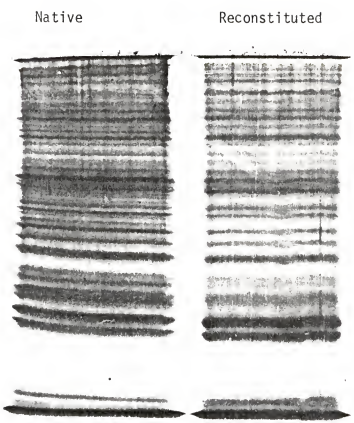

Figure 25. SDS polyacrylamide slab gel electrophoretic profiles of reconstituted and unreconstituted chromatin. One mg of G_1 DNA as chromatin was dissociated and mixed with 8 μg of the 0.4 M S phase SP-Sephadex fraction. Half of this chromatin was immediately treated with SDS and frozen. The other half was reconstituted and the reconstituted chromatin pellet was then treated with SDS.

is whether they are protein or nucleic acid in nature. While the lac operon of E. coli and the phage lambda are thought to be regulated by proteins, a number of investigators have postulated that nucleic acids are important in the regulation of at least some eucaryotic genes.[16,230-232] In eucaryotic cells there are a class of small RNAs which are found only in the nucleus[231,233,234] which are tissue specific[235-237] and which hybridize with repetitive DNA.[231,232,235-237] In their original model of eucaryotic gene expression Britten and Davidson[16] postulated that these are the regulators of transcription and more recently Brawerman[230] has postulated that these RNAs serve as primers for gene transcription. As was discussed in section B and as shown in Figure 5, centrifugation of the S phase nonhistone chromosomal proteins in CsCl does not diminish their ability to activate histone gene transcription from G_1 chromatin. If labeled nucleic acids are added to the chromosomal proteins this treatment has been shown to be able to remove at least 99.5% of the added nucleic acids.

While this would indicate that the ability to activate histone gene transcription is not due to the presence of free nucleic acid in the S phase chromosomal proteins, it does not rule out the possibility that the histone gene activator may be a nucleic acid-protein complex in which the components were very tightly, perhaps covalently, linked. The existence of such nucleic acid-protein complexes in the chromosomal proteins has been claimed by Huang and Huang[231] but this is controversial. (See McGilvery and Rickwood[238] for a review.) It is also interesting in this regard that if one examines the UV spectra of

the G-100 fraction shown in Figure 26, it has a 280/260 ratio of only 1.1. This may be due to a low content of aromatic amino acids, particularly tryptophan, but could also be due to the presence of approximately 2% nucleic acid. Since the histone gene activator may represent only a very small component of this fraction, it might contain a large proportion of nucleic acid. In order to approach this problem, 3 µg of labeled SP fraction was centrifuged in 5 M urea, 10 mM Tris (pH 8.3) containing 0.41 mg/ml CsCl for 80 hours at 37,500 rpm in a Beckman SW 50.1 rotor. The distribution of ^{3}H labeled SP fraction in this gradient, which is shown in Figure 27, was then compared with the distribution of histone gene activator activity. In order to assay for the ability to activate histone gene transcription, fractions 1 and 2, 3 through 5, 6 through 8 were pooled and 100 µl of each pool (containing 0.31, 0.05 and 0.01 µg of protein respectively) was reconstituted with 500 µg of G_1 DNA as chromatin. As shown in Figure 28, only transcripts from chromatin reconstituted with fractions 1 and 2 hybridized significantly with histone cDNA. These two fractions contain approximately 85% of the total protein on the gradient and have a density between 1.25 and 1.28 g/cc. These data are consistent with the histone gene activator being protein in nature but it should be noted that small amounts of nucleic acid would not be detected by this procedure. If a protein contained 5% nucleic acid its density would only be shifted approximately 0.03 g/cc.

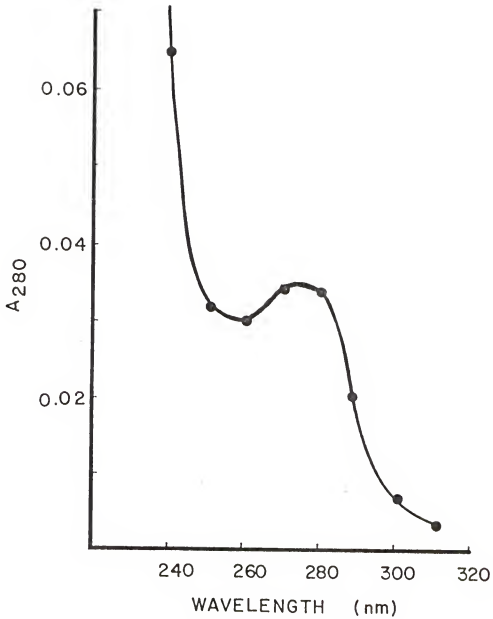

Figure 26. Ultraviolet spectra of the log phase G-100 fraction.

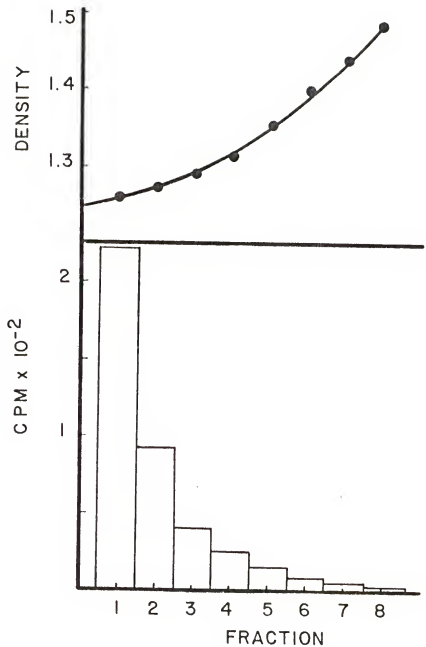

Figure 27. Centrifugation of S phase chromosomal proteins in CsCl. The ^{3}H-leucine labeled SP-Sephadex fraction of S phase chromosomal proteins was centrifuged for 80 hours at 37,500 rpm in a Beckman SW 50.1 rotor at 4^{o}C. The gradient was then fractionated and 100 µl of each fraction was counted. The upper panel shows the density at 4^{o}C of each fraction.

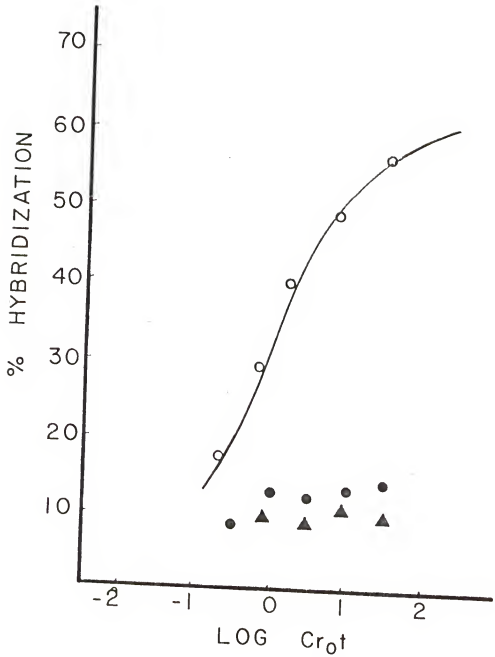

Figure 28. Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 chromatin reconstituted in the presence of fractions from CsCl density gradient containing S phase 0.4 M SP fraction. ^3H cDNA was annealed at 52°C to transcripts from 1 mg of G_1 DNA as chromatin reconstituted in the presence of 100 µl of fractions 1 and 2 (○), 3-5 (●), and 6-8 (△).

5. Digestion with Micrococcal Nuclease

In order to further examine whether the activation of histone gene transcription from G_1 chromatin by the S phase chromosomal protein fractions is due to the presence of nucleic acids in these preparations, the sensitivity of histone gene activator activity to digestion by micrococcal nuclease was determined. Micrococcal nuclease is a calcium dependent enzyme which can degrade RNA and both single and double stranded DNA.[239-241] As shown in Figure 29, the enzyme is active in 2 M urea, 0.1 M Tris, 1 mm $CaCl_2$ (pH 8.3) at 37°C and is capable under these conditions of degrading 18S ribosomal RNA and both single and double stranded calf thymus DNA. In addition to its broad specificity, an additional advantage of this enzyme is that since it is calcium dependent, it can be inactivated by the calcium specific chelating agent EGTA (Figure 29).

To determine the sensitivity of histone gene activator to micrococcal nuclease, 3.4 µg of the 0.4 M SP-Sephadex fraction in 1 ml of 2 M urea, 0.1 M Tris, 1 mM $CaCl_2$ (pH 8.3) was incubated for 30 minutes at 37°C with 0.06 µg of micrococcal nuclease. In a parallel reaction in which 3 µg of ^{3}H-labeled double stranded lambda DNA had been added to the same amount of the SP fraction, this amount of nuclease was sufficient to make 99% of the lambda DNA TCA soluble. In order to measure the ability of the nuclease treated SP fraction to activate histone gene transcription, EGTA was added to inactivate the enzyme and 0.3 µg of the nuclease treated SP fraction was reconstituted

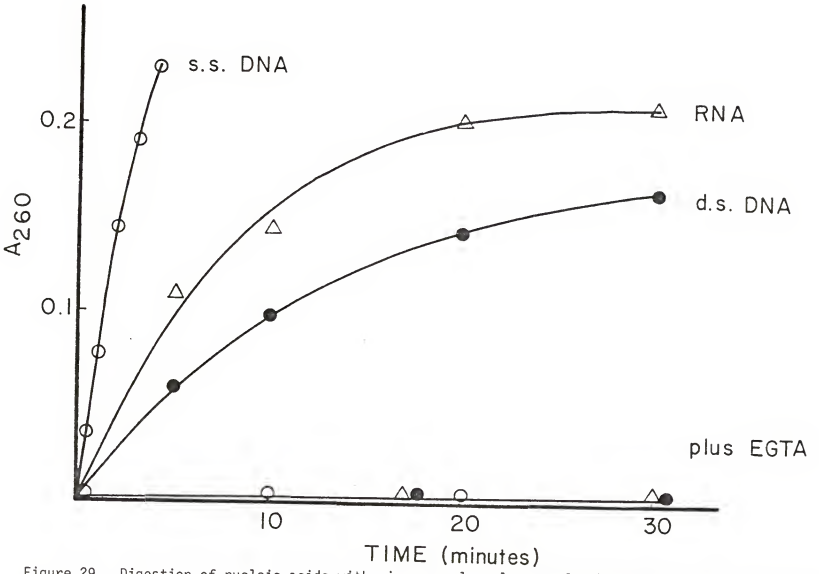

Figure 29. Digestion of nucleic acids with micrococcal nuclease. One hundred μg of ribosomal RNA (△) and both single (○) and double (●) stranded calf thymus DNA were digested with 0.06 μg of the enzyme and perchloric acid soluble material was measured. Some reaction mixtures contained 5 mM EGTA.

with 1 mg of G_1 DNA as chromatin. As is shown in Figure 30, there is no significant difference in the kinetics of hybridization with histone cNDA of transcripts from G_1 chromatin reconstituted in the presence of 0.3 μg of nuclease treated SP fraction and transcripts from G_1 chromatin reconstituted in the presence of 0.3 μg of untreated SP fraction.

To rule out the possibility that activation of histone gene transcription from G_1 chromatin by the nuclease treated SP fraction is due to the action of the nuclease or to the presence of EGTA, G_1 chromatin was reconstituted and then transcribed in the presence of EGTA and EGTA-inactivated micrococcal nuclease. As is also shown in Figure 30, transcripts from G_1 chromatin reconstituted in the presence of EGTA and EGTA-inactivated micrococcal nuclease do not hybridize with histone cDNA even at a Cr_0t of 30.

It should be noted that while these results indicate that the histone gene activator activity is resistant to micrococcal nuclease, it cannot be ruled out that the 0.4 M SP Sephadex fraction contains a small amount of nucleic acid which is important in the activation of histone gene transcription, but that this nucleic acid is nuclease resistant.

6. Activation of Histone Gene Transcription in Heterologous Systems[221]

Results from a number of laboratories indicate that histone proteins are very similar in different mammalian species and in different cell types of the same species.[56,61,242] While the

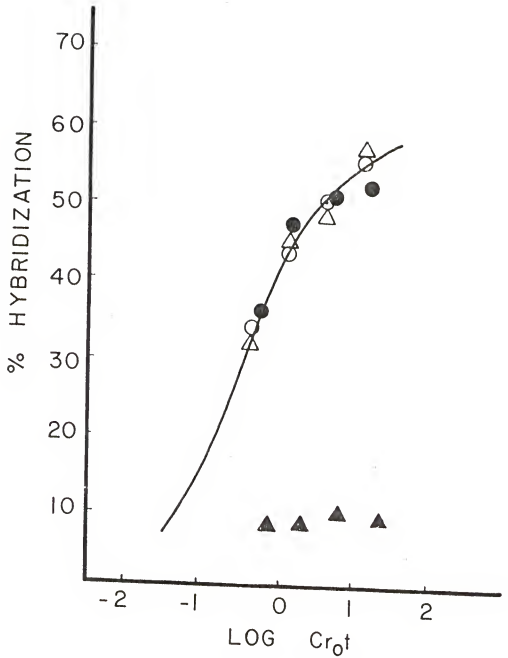

Figure 30. Kinetics of annealing of histone cDNA to in vitro transcripts from G_1 chromatin reconstituted with micrococcal nuclease treated chromosomal proteins. ^3H cDNA was annealed at 52°C to transcripts from 1 mg of G_1 DNA as chromatin reconstituted with 0.3 µg of nuclease treated (O) or untreated (●) 0.4 M SP fraction. G_1 chromatin was also reconstituted in the presence of EGTA and EGTA-inactivated micrococcal nuclease in the absence of additional chromosomal protein (▲) or with 0.3 µg of the 0.4 M SP-Sephadex fraction (△).

nonhistone chromosomal proteins have been shown to play a major role in the regulation of histone gene transcription in continuously dividing HeLa S_3 cells, it is not known whether the mechanism by which histone gene transcription is regulated is the same in other tissues and species. It is of particular interest to determine whether a highly transformed continuously dividing cell, such as HeLa, contains all of the components necessary to activate histone gene transcription from other cells which have greater degrees of growth control. To examine these questions chromatins from both contact-inhibited WI-38 human diploid fibroblasts and mouse liver have been dissociated and reconstituted in the presence of added chromosomal proteins from S phase HeLa cells.

a. Activation of histone gene transcription in contact-inhibited WI-38 cells

When WI-38 fibroblasts are grown to confluency in monolayer culture, less than 1% of the cells in the confluent monolayer continue to synthesize DNA. When the exhausted growth medium is replaced with fresh medium containing 20% fetal calf serum, an activation of DNA synthesis is evident at 10 hours and reaches a maximum at 12 hours following stimulation.[219,220,243] Using hybridization to a complementary DNA probe for the detection of histone mRNA sequences, Dr. Robert Jansing,[243] in this laboratory, has shown that histone mRNA becomes associated with polysomes during the activation of DNA synthesis and that the ability of chromatin from WI-38 cells to serve as a template for *in vitro* transcription of histone mRNA sequences also parallels

the onset of DNA replication. As shown in Figure 31, transcripts from chromatin of confluent WI-38 cells hybridize only to a limited extent with histone cDNA ($Cr_0t_{\frac{1}{2}}$ = 180)--such hybridization most likely being accounted for by the few cells which escape contact inhibition and continue DNA as well as histone synthesis.[243] In contrast, the kinetics of hybridization between histone cDNA and RNA transcripts from S phase (12 h) chromatin reveal a 500 fold activation of histone mRNA sequence transcription ($Cr_0t_{\frac{1}{2}} = 4 \times 10^{-1}$). This degree of activation is comparable to the increase in DNA synthesis in quiescent cells which have been stimulated to proliferate.

As is also shown in Figure 31, when chromatin from contact-inhibited WI-39 cells dissociated with 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) and is then reconstituted in the absence of additional chromosomal proteins, the same low level of hybrid formation between transcripts of this chromatin and histone cDNA was observed as is seen with transcripts of native, contact-inhibited WI-38 chromatin. In contrast, transcripts from chromatin of contact-inhibited WI-38 cells reconstituted in the presence of 100 µg of the 0.5 M QAE fraction of S phase HeLa chromosomal proteins (per mg WI-38 DNA as chromatin) hybridize with histone cDNA with a $Cr_0t_{\frac{1}{2}}$ of 4×10^{-1}. This degree of histone gene transcription is comparable to that observed when the same amount of contact-inhibited WI-38 chromatin is reconstituted in the presence of 1000 µg of S phase WI-38 chromosomal proteins and that seen from native chromatin of WI-38 cells which have been stimulated to proliferate.

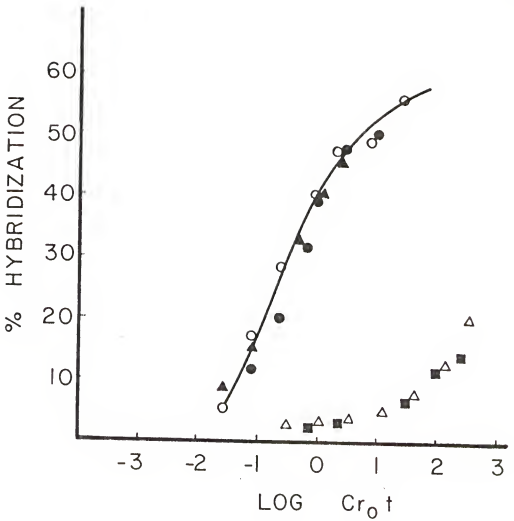

Figure 31. Kinetics of annealing of histone cDNA to in vitro transcripts from chromatin of contact-inhibited WI-38 fibroblasts reconstituted in the presence of chromosomal proteins from S phase HeLa cells or from WI-38 cells which have been stimulated to proliferate. ^{3}H cDNA was annealed at 52^{o}C to transcripts from 1 mg of DNA as chromatin from contact-inhibited WI-38 fibroblasts reconstituted with no additional chromosomal proteins (Δ), 100 µg of the 0.5 M QAE fraction of S phase HeLa chromosomal proteins (O), or 1000 µg of total chromosomal proteins from S phase WI-38 cells (●). ^{3}H cDNA was also annealed to transcripts from native chromatin of contact-inhibited WI-38 fibroblasts (■) or from WI-38 fibroblasts which had been stimulated to proliferate (▲).

b. Activation of histone gene transcription from mouse liver chromatin

As shown in Figure 32, when chromatin is isolated from mouse liver and is transcribed in vitro, the transcripts do not hybridize significantly with HeLa histone cDNA even at a Cr_0t of 30. When this chromatin is dissociated with high salt and urea and is then reconstituted in the absence of additional chromosomal protein, again no significant hybrid formation between transcripts and HeLa histone cDNA is observed. In contrast, when mouse liver chromatin is reconstituted in the presence of 1000 µg of total chromosomal proteins from S phase HeLa cells per mg of mouse liver chromatin DNA, the amount of mouse histone mRNA sequences transcribed is increased over 1000 fold ($Cr_0t_{\frac{1}{2}} = 4 \times 10^{-1}$).

To rule out the possibility that endogenous HeLa RNA associated with the S phase chromosomal protein at least in part accounts for the formation of hybrids with HeLa histone cDNA, mouse liver chromatin which had been reconstituted in the presence of 1000 µg of HeLa chromosomal protein was placed in the transcription mixture without the addition of RNA polymerase, and the endogenous RNA was extracted by the same procedure used for the isolation of in vitro transcripts. When this endogenous RNA is annealed with HeLa histone cDNA, there is no significant hybrid formation (Figure 32). That hybridization of histone cDNA to transcripts from mouse liver chromatin reconstituted in the presence of S phase HeLa chromosomal proteins is not due to endogenous HeLa sequences is also suggested by the fact that the S phase

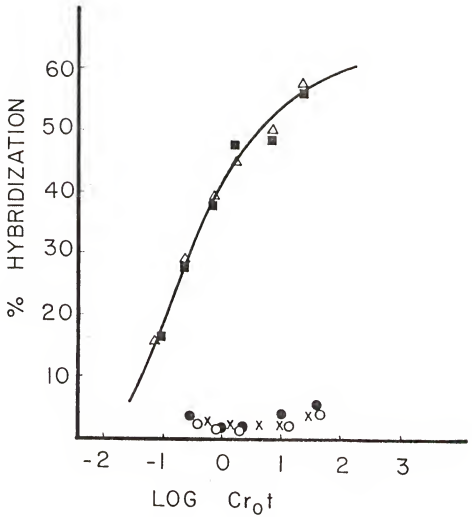

Figure 32. Kinetics of annealing of histone cDNA to in vitro transcripts from mouse liver chromatin, or mouse liver chromatin reconstituted in the presence of S phase HeLa chromosomal proteins. ^{3}H cDNA was annealed at $52^{o}C$ to transcripts from native mouse liver chromatin (O), or transcripts from 1 mg of mouse liver DNA as chromatin reconstituted in the presence of no additional protein (●), 1000 µg of total S phase HeLa chromosomal proteins (△), 100 µg of the 0.5 M NaCl QAE fraction of the S phase HeLa chromosomal proteins (■), or 100 µg of the 0.25 M NaCl QAE fraction of the S phase HeLa chromosomal proteins (X).

HeLa chromatin from which these proteins are isolated contains only very low levels of endogenous histone sequences. Although the amount of endogenous histone sequences varies, we generally find less than 10% hybrid formation at a Cr_0t of 100 between histone cDNA and endogenous RNA from S phase chromatin isolated either with or without the addition of carrier RNA. Additionally, it should be pointed out that the HeLa S phase chromosomal proteins contain only very small amounts of nucleic acids (0.2% or less) as estimated by the ratio of absorbance at 280 and 260 nm and by pulse and long-term labeling with ^{3}H-thymidine.

When 1 mg of mouse liver DNA as chromatin is dissociated with high salt and urea, reconstituted in the presence of 1000 µg of total S phase HeLa chromosomal proteins or 100 µg of the 0.5 M QAE fraction of these proteins, and then transcribed in vitro with E. coli RNA polymerase, 80-100 µg of RNA can be recovered as purified transcripts. When these mouse liver RNAs are annealed to histone cDNA, as shown in Figure 32, they both hybridize with a $Cr_0t_{\frac{1}{2}}$ of approximately 4×10^{-1}. In contrast, as is also shown in Figure 32, when 1 mg of mouse liver DNA as chromatin is reconstituted in the presence of 100 µg of the 0.25 M NaCl QAE fraction of the HeLa S phase chromosomal protein, even though approximately the same amount of RNA is transcribed, the transcripts do not show significant hybrid formation with histone complementary DNA.

In Figure 33, the melting profile of the hybrids formed between HeLa histone cDNA transcripts from mouse chromatin reconstituted in the presence of either 1000 µg of total S phase chromosomal proteins

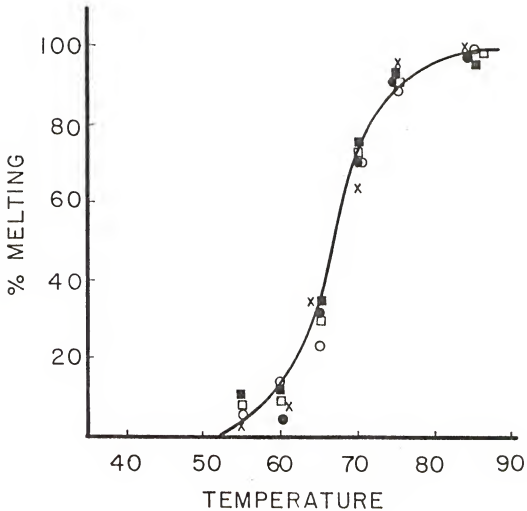

Figure 33. Melting profile of hybrids formed between histone cDNA and histone mRNA (□), between histone cDNA and transcripts from 1 mg of DNA as chromatin from mouse liver chromatin reconstituted in the presence of 1000 µg of total S phase chromosomal proteins (○), or 100 µg of the 0.5 M NaCl QAE-Sephadex fraction of S phase HeLa chromosomal proteins (●), between histone cDNA and transcripts from 1 mg of DNA as chromatin from contact-inhibited WI-38 fibroblasts reconstituted in the presence of 100 µg of the 0.5 M NaCl fraction of S phase HeLa chromosomal proteins (X), and between histone cDNA and polysomal RNA from mouse L-929 cells (■). The $Cr_0t_{\frac{1}{2}}$ of the hybridization reaction between histone cDNA and mouse L-929 cell polysomal RNA under these conditions is 7.4, which is consistent with this RNA containing approximately 0.23% histone sequences.

or 100 μg of the 0.5 M QAE fraction of the S phase chromosomal proteins (per mg mouse liver chromatin DNA) is shown. Within our limits of detection, there are no reproducible differences in the melting profiles of the hybrids formed between HeLa histone cDNA and HeLa histone mRNA, mouse L929 cell polysomal RNA, and transcripts from mouse liver or WI-38 fibroblast chromatin reconstituted in the presence of chromosomal proteins. It should be noted that small T_m differences would not be detected by this procedure.

c. Effect of chromosomal proteins from S phase HeLa cells on transcription of mouse globin sequences

Fetal liver is an erythropoetic organ which contains globin mRNA sequences, and Gilmour and Paul[115] have found that chromatin from fetal mouse liver will serve as a template for the *in vitro* transcription of globin mRNA sequences by *E. coli* polymerase. In contrast, as has been shown previously in birds[116] and is shown in Figure 34 for mouse, globin mRNA sequences are not transcribed *in vitro* by *E. coli* RNA polymerase from chromatin of adult liver, which is not an erythropoetic tissue.

In order to rule out the possibility that chromosomal proteins from S phase HeLa cells make the histone genes of mouse liver chromatin available for transcription nonspecifically, we have examined the effect of HeLa chromosomal protein on the transcription of globin sequences from mouse chromatin. As discussed above, when 1 mg of DNA as chromatin from adult mouse liver is dissociated and then reconstituted

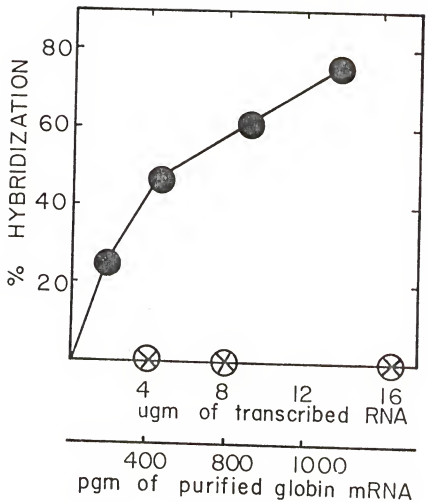

Figure 34. Kinetics of annealing of globin cDNA to in vitro transcripts from mouse liver chromatin. Globin cDNA was annealed to transcripts from native mouse liver chromatin (X) and from mouse liver chromatin dissociated and reconstituted in the presence of the 0.5 M NaCl QAE-Sephadex fraction from S phase HeLa S_3 cells (135 µg of protein added/mg of DNA as chromatin) (○). Control experiments to ensure that there were no inhibitors of hybridization in the chromatin transcripts were performed as follows: A known quantity of authentic mouse globin mRNA was mixed with chromatin transcript RNA, and the mixture was incubated with globin cDNA (●). The presence of chromatin transcript RNA did not interfere with hybridization of the mouse globin mRNA.

in the presence of 1000 μg of S phase total HeLa chromosomal proteins or 100 μg of the 0.5 M fraction of the HeLa chromosomal proteins, the amount of histone mRNA sequences detected in the transcripts is increased over 1000 fold from that found in transcripts from native mouse liver. Significant differences in the overall template activities of these reconstituted chromatin preparations are not observed. As shown in Figure 34, these same transcripts from mouse liver chromatin reconstituted with the 0.5 M NaCl fraction of S phase HeLa chromosomal proteins show no significant hybrid formation with mouse globin complementary DNA. If a mixture of globin mRNA and transcripts from mouse liver chromatin is hybridized with globin cDNA, the globin mRNA anneals with the globin cDNA as expected. Globin mRNA sequences would have been detected in the chromatin transcripts had they been present in as little as 1 part per 10^5.[244] The globin mRNA hybridization analysis was carried out by Dr. Jeffery Ross of the University of Wisconsin.

SUMMARY AND CONCLUSIONS

In initial studies in this laboratory[13,199] it was shown that when chromatin isolated from S phase HeLa cells is transcribed *in vitro* with *E. coli* RNA polymerase, its transcripts hybridize with histone cDNA with a $Cr_0t_{1/2}$ of 2×10^{-1} (compared to a $Cr_0t_{1/2}$ of 1.7×10^{-2} for the histone mRNA-cDNA reaction). That the histone sequences present in these S phase transcripts are due to new synthesis *in vitro* is suggested by the lack of hybrid formation with histone cDNA of endogenous RNA isolated from S phase chromatin with or without the addition of *E. coli* RNA as a carrier. In contrast to transcripts from S phase chromatin, transcripts from chromatin isolated from G_1 phase cells do not hybridize with histone cDNA even at a Cr_0t of 100. That histone sequences would have been detected if they had been transcribed is suggested by the fact that histone mRNA added to G_1 chromatin before *in vitro* transcription can subsequently be detected in the purified transcripts. It was also shown that incubation at 100°C for 10 minutes to dissociate any pre-existing hybrids has no effect on the hybridization of histone cDNA and transcripts from G_1 chromatin. These results indicate that while the total amount of RNA transcribed from G_1 and S phase cells is similar, histone sequences are efficiently transcribed from chromatin isolated from S phase cells, but that chromatin isolated

from G_1 phase cells does not serve as a template for the transcription of histone genes. This cell cycle stage specific transcription of histone sequences is consistent with the tissue specific transcription from chromatin by E. coli RNA polymerase which has been observed for the globin[115,116] and ovalbumin genes.[14,225] The maximal hybridization with histone cDNA and the melting temperature of the hybrids formed are the same for S phase transcripts and histone mRNA isolated from the polysomes of S phase cells, but at present very little is known about how the accuracy and specificity of initiation, termination and DNA strand selection in vitro compare with those seen in vivo. It should also be stressed that it is not known whether the mechanisms which regulate in vitro transcription of the histone sequences from chromatin by a bacterial polymerase are the same as those primarily responsible for the changes in histone gene transcription during the cell cycle.

Other early studies[13] were directed toward determining which component of chromatin is responsible for the difference in in vitro transcription of histone sequences from G_1 and S phase chromatin. In these studies both G_1 and S pahse chromatin were dissociated and fractionated into DNA, histone and nonhistone chromosomal proteins. Pooled DNA and histones were then reconstituted with either G_1 or S phase nonhistone chromosomal proteins. Even though the total amount of RNA transcribed from chromatin reconstituted with G_1 or S phase nonhistone chromosomal proteins is similar, the RNA transcribed from chromatin reconstituted with S phase nonhistone chromosomal proteins

hybridized with histone cDNA with the same kinetics of hybridization as the transcripts from native S phase chromatin, whereas the RNA transcribed from chromatin reconstituted with G_1 nonhistone chromosomal proteins--like the RNA transcribed from native G_1 chromatin--did not show significant hybrid formation with histone DNA. These results are consistent with the nonhistone chromosomal protein portion of chromatin being responsible for the differences in transcription of histone sequences in G_1 and S phase chromatin. Such a regulatory role for the nonhistone chromosomal proteins is in agreement with the results of several laboratories which have indicated that the nonhistone chromosomal proteins are responsible for the tissue specific transcription of globin genes[10,11,12,117] and the hormone induced transcription of the ovalbumin genes.[14,225] These results, however, do not indicate whether the difference in in vitro transcription of the histone sequences from G_1 and S phase chromatin is due to an activator of histone gene transcription present in S phase nonhistone chromosomal proteins, repressor of histone gene transcription present in the G_1 chromosomal proteins or both.

In order to approach this problem, chromatin from G_1 and S phase cells was dissociated and then reconstituted in the presence of additional chromosomal proteins.[226] When chromatin from G_1 phase cells is dissociated and then reconstituted in the presence of increasing amounts of S phase total chromosomal proteins, hybrid formation between transcripts from these reconstituted chromatins and histone cDNA occurs at progressively lower Cr_ot values suggesting that histone

sequences are being made available for transcription. This apparent stimulation of histone gene transcription is not observed when G_1 chromatin is dissociated and then reconstituted in the absence of additional chromosomal proteins or when G_1 chromatin is reconstituted in the presence of additional G_1 phase chromosomal proteins. In order to determine which component of the S phase chromosomal proteins is responsible for this activation of histone gene transcription from G_1 chromatin, S phase chromosomal proteins were fractionated into histone and nonhistone chromosomal proteins with QAE-Sephadex. When these fractions were assayed for their ability to activate histone gene transcription, it was found that only the nonhistone chromosomal protein fraction can activate histone gene transcription from G_1 chromatin. When the reciprocal experiment was performed in which S phase chromatin was dissociated and then reconstituted in the presence of additional G_1 phase chromosomal proteins there was no effect on histone gene transcription, even at a one-to-one ratio of additional G_1 protein to DNA. These results support the contention that the difference in *in vitro* transcription of histone sequences from G_1 and S phase chromatin is due to the nonhistone chromosomal protein portion of chromatin and show that this difference can be accounted for by a component(s) of the S phase nonhistone chromosomal proteins that has the ability to activate histone gene transcription from G_1 phase chromatin in a dose dependent fashion. Such activation of histone gene transcription by the nonhistone chromosomal proteins is consistent with that seen by Paul *et al.*[10] for the globin gene and the recent result of Tsai *et al.*[225] with the

ovalbumin gene. It should be stressed that while the overall mechanism is positive in nature, the S phase nonhistone chromosomal proteins may act as derepressors which override specific or nonspecific repressors of histone gene transcription present in G_1 chromatin. Such an interpretation is consistent with the findings of Stein et al.[245] that histone sequences are efficiently transcribed from deproteinized DNA and that this transcription is inhibited by histones (although to the same degree as they inhibit total RNA transcription). It should also be pointed out that there may be other perhaps specific, regulatory molecules that affect histone gene transcription that have been lost or inactivated during chromatin isolation, fractionation, or reconstitution. There may be also other perhaps more subtle factors which interact with the homologous RNA polymerase or which act in the intact cell.

To determine which component of the S phase nonhistone chromosomal proteins is responsible for the ability of these proteins to activate histone gene transcription, the S phase chromosomal proteins were fractionated using ion exchange and gel filtration chromatography.[227] When total S phase chromosomal proteins were fractionated in 5 M urea at pH 8.3 on QAE-Sephadex, the histones and approximately 10% of the nonhistone chromosomal proteins were not bound and were eluted in the void volume, whereas a complex but electrophoretically distinct group of nonhistone chromosomal proteins could be eluted by 0.1, 0.25, 0.50 and 3 M NaCl. When these fractions were assayed for their ability to activate histone gene transcription, only the fraction

eluted 0.5 M NaCl was found to be active. The 0.5 M QAE fraction was then further fractionated using the strong cation exchanger SP-Sephadex. When the proteins were chromatographed on this column at pH 5.2, histone gene activator activity was found only in the material eluted with 0.4 M NaCl. The SP-Sephadex fraction was then further purified by chromatography on Sephadex G-100. While the purified fraction is not homogenous, it contains only 0.04-0.06% of the total chromosomal proteins but has sufficient ability to activate histone gene transcription to account for all the activity present in the total chromosomal proteins.[227]

Since chromatography on gel filtration resins such as Sephadex G-100 is a function of molecular size, this technique can be used to determine apparent molecular weights. When the S phase total chromosomal proteins were chromatographed on Sephadex G-100 in 3 M NaCl, 5 M urea, 10 mM Tris (pH 8.3) only the material eluting with an apparent molecular weight of 40,000-60,000 daltons had the ability to activate histone gene transcription from G_1 chromatin.[227] Comparable results were obtained when the 0.4 M SP fraction was chromatographed on this resin. In this experiment only the material which eluted with an apparent molecular weight of 45,000-80,000 daltons had the ability to activate histone gene transcription.[227] It should be emphasized, however, that these are only apparent molecular weights on Sephadex G-100. If the protein are not approximately spherical or if they interact with other proteins or with the Sephadex, the actual molecular weight can be very different.

In order to determine whether the histone gene activator is protein or nucleic acid in nature, the S phase chromosomal proteins were subjected to centrifugation in CsCl in the presence of 5 M urea.[226] This procedure very effectively removes nucleic acids from the chromosomal proteins but does not diminish the ability of these proteins to activate histone gene transcription from G_1 chromatin. While these results would indicate that the ability to activate histone gene transcription is not due to the presence of free nucleic acid in the S phase chromosomal proteins, it does not rule out the possibility that histone gene activator may be a nucleic acid-protein complex. In order to approach this question, the 0.4 M SP-Sephadex fraction of the S phase chromosomal proteins was subjected to buoyant density centrifugation in CsCl in the presence of 5 M urea under conditions in which nucleic acids would be pelleted. The distributions of protein and histone gene activator activity over the gradient were then compared.[227] The results of this experiment indicate that 85% of the protein and all of the histone gene activator activity were present in the top 2 fractions of the gradient (density 1.25-1.28 g/ml). While this is consistent with the histone gene activator being protein in nature, it does not rule out the possibility that it might contain as much as 5% nucleic acid.

In order to further examine whether the ability of the S phase chromosomal protein fractions to activate histone gene transcription is due to the presence of nucleic acids in these preparations, the sensitivity of the histone gene activator activity to digestion with micrococcal nuclease was determined. Micrococcal nuclease can degrade

RNA and both single and double stranded DNA, but is inactivated by the calcium specific chelating agent EGTA. In order to determine the sensitivity of the histone gene activator activity to this enzyme, the 0.4 M SP-Sephadex fraction of S phase chromosomal proteins was incubated with a sufficient amount of the enzyme to degrade at least 99% of the free nucleic acid present. The enzyme was then inactivated with EGTA and the nuclease treated SP fraction was reconstituted with G_1 chromatin. When the reconstituted chromatin was transcribed and the purified transcripts were annealed with histone cDNA, it was found that there were no significant differences in the kinetics of annealing with histone cDNA of transcripts from G_1 chromatin reconstituted in the resence of 0.3 µg of nuclease treated SP fraction and transcripts from G_1 chromatin reconstituted in the presence of 0.3 µg of untreated SP fraction. The possibility that the activation of histone gene transcription is due to the action of the nuclease or the presence of EGTA and EGTA-inactivated micrococcal nuclease does not transcribe histone sequences. These results indicate that the histone gene activator activity is resistant to micrococcal nuclease digestion but this resistance does not conclusively show that nucleic acids are not involved in the activation of histone gene transcription since the SP-fraction may contain nucleic acids which are not degraded by the enzyme either because of their structure, or more likely, because of their association with proteins or other molecular species.

In addition to G_1 chromatin, it was also shown that the S phase HeLa chromosomal proteins can activate histone gene transcription from

chromatin of both quiescent WI-38 fibroblasts and mouse liver.[221] These results would indicate that not only are the histone proteins very similar in different mammalian species and in different tissues of the same species but also that the mechanisms by which in vitro transcription of the histone genes is regulated by the nonhistone chromosomal proteins in HeLa cells, WI-38 cells, and mouse liver may be the same or similar. This can be accounted for by postulating that the DNA sequences with which certain nonhistone chromosomal proteins interact, perhaps regulatory sequences, are conserved between mouse and human. Alternatively the DNA sequences involved with activation of histone gene transcription in vitro may differ between mouse and human, but both types or sequences may be recognized by the HeLa nonhistone proteins. However, our results do illustrate that a highly transformed continuously dividing cervical carcinoma cell, such as HeLa, contains all of the components necessary to make the histone genes of contact-inhibited tissue culture cells or nondividing cells from an intact organism available for transcription in vitro by E. coli RNA polymerase.

The activation of histone gene transcription from mouse liver by the S phase chromosomal proteins also provides a way of more carefully examining the specificity of the histone gene activator. Fetal mouse liver is an erythropoetic organ and it has been shown that globin mRNA sequences can be transcribed from fetal mouse liver chromatin by E. coli RNA polymerase.[115] In contrast, globin mRNA sequences are not transcribed in vitro by E. coli polymerase from chromatin of adult liver (which is not erythropoetic).[221] When mouse

liver chromatin is reconstituted in the presence of S phase chromosomal proteins the amount of histone sequences transcribed can be increased over 1000 fold from that seen in transcripts from native mouse liver. Reconstitution in the presence of S phase chromosomal proteins, however, does not significantly change the total template capacity of the chromatin and does not make the mouse globin genes available for transcription.[239] While this does not indicate that the histone gene activator is necessarily specific only for the histone genes it does indicate that it does make the sequences available for transcription randomly.

Taken together these results provide direct evidence that the nonhistone chromosomal proteins can regulate the expression of specific eucaryotic genes and indicate that the differences in histone gene transcription between G_1 and S phase chromatin can be accounted for by a component or components of the S phase nonhistone chromsomal proteins that has the ability to activate histone gene transcription from G_1 chromatin. The dose dependent activation of histone gene transcription from G_1 chromatin by the S phase chromosomal proteins provides a quantifiable assay which has been used to monitor the partial purification of the histone gene activator and to characterize it with respect to its apparent molecular weight on Sephadex G-100, density in CsCl, sensitivity to micrococcal nuclease digestion, and its ability to activate histone gene transcription in heterologous systems. While much remains to be learned about the molecular mechanism by which it activates histone gene transcription, this assay provides a tool which can be used in its further purification and characterization.

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BIOGRAPHICAL SKETCH

The author was born in Columbia, South Carolina, September 16, 1951. He attended elementary and secondary schools in the Columbia area and graduated from Brookland Cayce High School.

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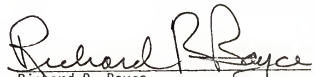

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